A STUDY OF SELECTED ISOENZYMES IN SHEEP

By

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This thesis has been composed by me and describes my own work. It has not been submitted in any form to any other University.

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SUMMARY

The work described in this thesis involved a study of isoenzymes (multiple forms of enzymes arising from genetically determined differences in primary structure) in ovine tissues and serum. The study is divided into three major parts.

The first part describes the technical problems encountered and the methods used to solve these problems in relation to separating the isoenzymes of lactate dehydrogenase, glucosephosphate isomerase, aldolase, aspartate aminotransferase and creatine kinase. Both isoelectric focusing and electrophoresis were investigated but only the latter proved suitable for the quantitative estimations described in parts two and three.

In the second part, normal isoenzyme levels were established in ovine tissues and an extensive investigation, involving 151 sheep, was carried out to determine the effect of physiological parameters on serum isoenzyme levels. Time after parturition in ewes and age in lambs affected serum lactate dehydrogenase isoenzyme levels but sex and growth rate produced no effects of any clinical significance. In a separate experiment, the frequency and mode of inheritance of two genetic variants of glucosephosphate isomerase was investigated.

In the third part, the serum and tissue levels of lactate dehydrogenase and creatine kinase were studied in respiratory and gastrointestinal tract disease. In chronic proliferative exudative pneumonia and in acute pasteurella pneumonia, no changes in serum isoenzyme levels were observed which were likely to be of diagnostic value, despite a marked increase in the total lactate dehydrogenase level and changes in the distribution of its isoenzymes in pneumonic lungs. In chronic gastrointestinal parasitism, changes in serum lactate dehydrogenase and creatine kinase isoenzyme levels attributable to damage to the intestinal tract caused by Trichostrongylus vitrinus were detectable and may be of value in following the course of experimental infections in the live sheep. In the acute and chronic stages of parasitic gastritis caused by Ostertagia circumcincta or Haemonchus contortus, no diagnostically useful serum isoenzyme changes were observed. In acute O. circumcincta infection, isoenzyme levels in serum, and lymph from the gastric lymph duct were measured. Lymph isoenzymes were no more sensitive than serum isoenzymes in detecting damage to the abomasal mucosa, suggesting that the isoenzymes may have been released from the mucosa into the lumen of the abomasum rather than into the circulation via the lymphatic drainage.

Changes in the lactate dehydrogenase isoenzyme distribution of the abomasal mucosa were observed in acute parasitic gastritis but these were not reflected in the serum isoenzyme levels.

STATISTICAL DEFINITIONS

n = number of samples

$\bar{x}$ = mean value

$\bar{\Delta}$ = mean difference between observations

s = standard deviation

t = t value

F = F value

df = degrees of freedom

p = level of significance

CV = coefficient of variation

a = distance above the baseline at which a regression line cuts the y - axis

b = regression coefficient

r = correlation coefficient

INTRODUCTION

INTRODUCTION

An enzyme is a protein with catalytic properties due to its power of specific activation (Dixon and Webb, 1958).

The existence of enzymes was first clearly demonstrated by Buchner in 1897 who showed that yeast extract, free of living cells, could produce alcohol from sugar. Since the purification of the first enzyme in a crystalline form in 1926, there have been tremendous advances in the knowledge of pathways of metabolism and the enzymes involved in those pathways.

The study of enzyme activity in serum as a means of determining the site, severity and duration of tissue damage, in both human and veterinary medicine, has aroused considerable interest in the last few decades, since it became apparent that when cells are damaged in the course of a disease, many intracellular enzymes are released into the circulation.

Enzymes in the blood are derived from a variety of sources and can be classified as follows (after Bergmeyer, 1974) -

(a) Plasma-specific enzymes e.g. prothrombin, plasminogen

(b) Secreted enzymes e.g. pancreatic alpha-amylase, pepsinogen

(c) Cellular enzymes (enzymes of tissue metabolism) e.g. lactate dehydrogenase, alanine aminotransferase, fructose-diphosphate aldolase.

A reduction in the level of enzymes may occur due to failure of enzyme synthesis in damaged cells (Mattenheimer, 1971), but in the majority of cases, serum enzyme levels increase in cellular damage.

The extent of this detectable increase in the level of an enzyme in the serum due to cellular damage depends on:-

(a) the number of cells damaged
(b) the amount of enzyme present in the cell
(c) the intracellular site of the enzyme
(d) the degree of loss of integrity of the cell membrane and cell organelles
(e) the presence or absence of binding of the enzyme to cell structures
(f) the half-life of the enzyme in the serum

The amount of enzyme in the cell is of particular importance. Very few enzymes are found in only one tissue, although they may be present at higher levels in some tissues than others. It is this lack of tissue specificity which is one of the major limitations of studying total serum enzyme levels.

In man, a limited number of serum enzymes are

regarded as being organ specific. Thus, sorbitol dehydrogenase and ornithine carbamoyltransferase are indicative of liver disease (Mattenheimer, 1971) whereas phospholipase A indicates acute pancreatitis (Dreiling and Greenstein, 1970).

In dogs and cats, a few enzymes, used singly, can be considered to be reasonably specific to certain tissues e.g. alanine aminotransferase from the liver. Usually, however, it is more useful to assay several enzymes to detect a tissue-specific total enzyme pattern in the serum.

In cattle and sheep, tissue specificity in serum enzymes is almost non-existent, although some occur predominantly in one organ e.g. sorbitol dehydrogenase and glutamate dehydrogenase in the liver.

This lack of tissue specificity has limited the diagnostic usefulness of clinical enzymology and recently, attention has been turned from total enzyme estimations to the study of isoenzymes in the hope of improving this situation.

Isoenzymes are multiple forms of enzymes arising from genetically determined differences in primary structure (IUPAC - IUB Commission on Biochemical Nomenclature, 1976).

In man some isoenzymes appear to be specific to one tissue e.g. acid phosphatase from red blood cells and acid phosphatase from prostate gland, and their

presence in serum is a definitive indicator of their release from these tissues. In other cases, such as lactate dehydrogenase, more than one isoenzyme is present in many tissues, the proportion of the total activity contributed by each isoenzyme varying with the tissue of origin.

Serum lactate dehydrogenase isoenzyme levels have been used in preference to total lactate dehydrogenase for the detection of damage to skeletal muscle (Yasmineh et al., 1978), myocardium (Latner and Skillen, 1961) and liver (Hess and Walter, 1961) in man.

Similarly, in the dog and horse, certain alkaline phosphatase isoenzymes have been shown to indicate damage to specific organs, for example the level of the bone isoenzyme is increased in the serum in osteosarcoma in the dog (Rogers, 1976) and the intestinal isoenzyme is increased in intestinal cellular disruption in the horse (Blackmore and Palmer, 1977). In the horse, lactate dehydrogenase isoenzymes have been used to indicate damage to skeletal muscle, myocardium and liver (Coffman, 1974) and creatine kinase isoenzymes to indicate skeletal muscle damage (Johnson and Perce, 1981).

In ruminants, isoenzyme investigations have been almost confined to alkaline phosphatase and lactate dehydrogenase. Total serum alkaline phosphatase is

very variable in ruminant serum (Allcroft and Folley, 1941), while the intestinal isoenzyme varies with diet and blood group (Healy and Davis, 1975) making the enzyme unreliable as a diagnostic aid. Serum lactate dehydrogenase isoenzymes have been used to indicate skeletal and cardiac muscle necrosis in calves (Dotta and Pellegrino, 1972), skeletal muscle damage in sheep (Paulson, Pope and Baumann, 1966) and liver damage in cattle (Keller, 1974b) and sheep (Michálek, and Vodrážka, 1977). There are few reports of the use of lactate dehydrogenase isoenzymes in ruminants for detecting damage to tissues other than muscle or liver.

The results obtained in investigations such as these are affected by the method employed to study the isoenzymes. There are a variety of ways of separating isoenzymes, some being more reliable than others.

The most convenient and extensively used method of separating the isoenzymes of a particular enzyme in a complex mixture such as a tissue homogenate or serum is by electrophoresis, followed by location of the pattern of isoenzyme bands in the electrophoresis medium by an enzyme-specific reaction, usually resulting in the production of a coloured compound. However, the use of isoelectric focusing allows further investigations to be made into the differences in isoenzyme pattern between tissues.

In many cases, when isoenzymes are separated by

isoelectric focusing, they further subdivide so that each isoenzyme produces a complex band pattern. Thus, an isoenzyme which is present in two tissues, and has the same electrophoretic mobility in both tissues, may show a tissue-specific pattern when subjected to isoelectric focusing, for example, lactate dehydrogenase isoenzyme 5(LDH_5) from skeletal muscle produces a different pattern on focusing to LDH_5 from rumen mucosa. If these tissue-specific patterns could be demonstrated in serum, the value of isoenzymes in the diagnosis of disease would be markedly improved.

With these aims in view, I embarked on the work to be described in this thesis. The main objectives were to investigate methods of isoenzyme separation and to determine whether sufficiently tissue-specific isoenzyme patterns could be detected in sheep to enable these patterns to be used as indicators of tissue damage, particularly to organs such as lung, abomasum and small intestine, since no effective biochemical tests are available for detecting damage to these sites.

CHAPTER 1

SELECTION OF ENZYMES FOR STUDY

A large number of enzymes exist as multiple forms but in only a very few cases is a serum isoenzyme pattern obtained which is a specific indicator of damage to a single organ (e.g. the appearance of prostatic acid phosphatase in human prostatic carcinoma). It was therefore decided to select several enzymes whose isoenzyme patterns in the serum could be studied in combination, to indicate the site of tissue damage. I was particularly interested in studying tissues such as the lung, in which it is at present difficult to detect tissue damage.

The following enzymes were considered for inclusion in this study.

Hexokinase[1] (ATP: D-hexose 6-phosphotransferase[2], E.C.2.7.1.1[3]), which catalyses the conversion of glucose to glucose-6-phosphate in the first step of the glycolytic pathway (see Fig. 1.1), is known to have four isoenzymes. Types I to III have a wide tissue distribution (Rogers, Fisher and Harris, 1975) while type IV is almost specific to the liver. The latter can only be demonstrated after electrophoresis in the presence of high concentrations of its substrate glucose but since type III is inhibited by high levels of glucose the isoenzymes cannot be demonstrated simultaneously.

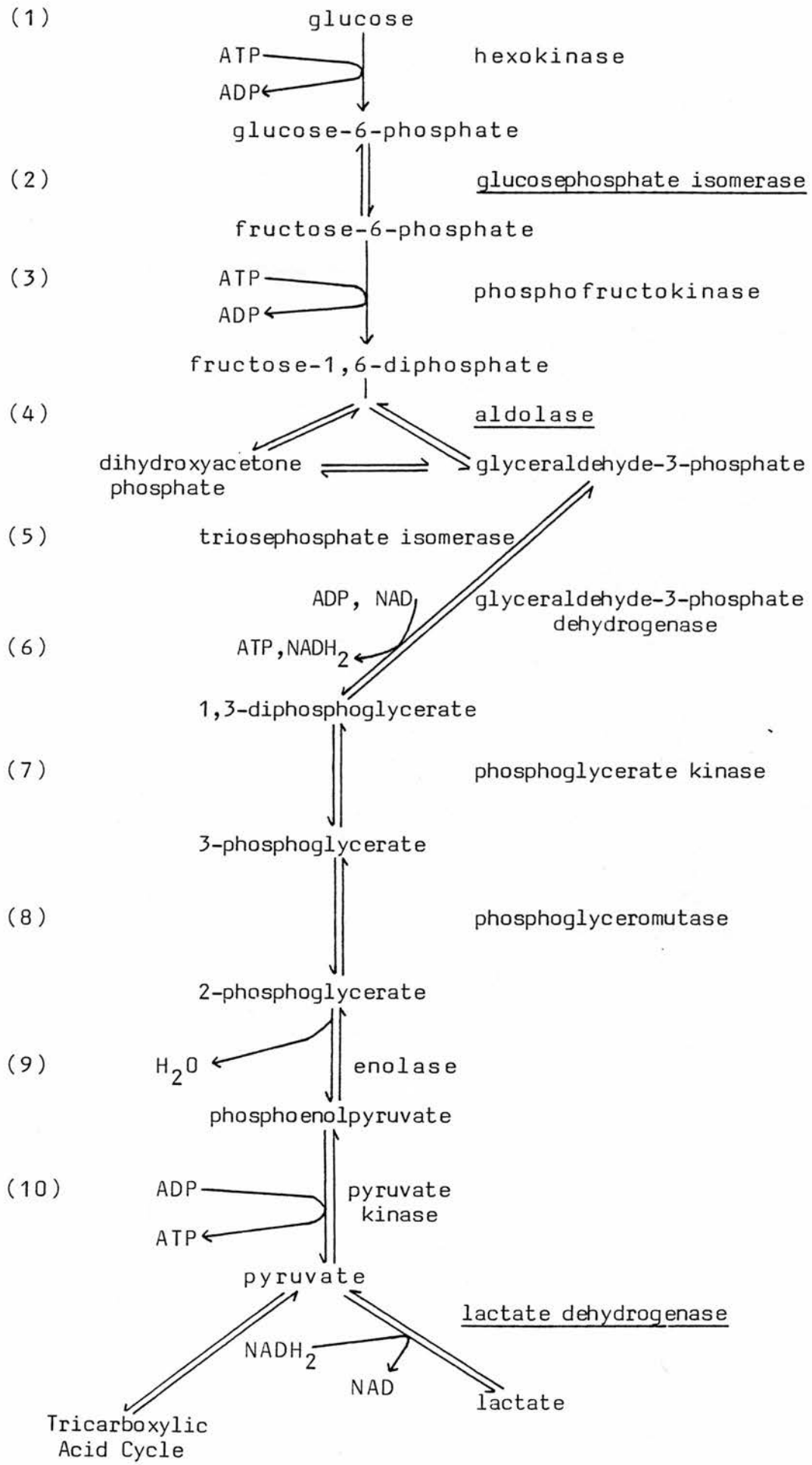
1 Trivial name, 2 Systematic name, 3 Enzyme Commission number.

The tissue distribution of the isoenzymes of glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), E.C.1.4.1.3), which brings about the oxidation of glutamic acid to ∝-oxoglutaric acid has been studied in human tissues by Nelson et al. (1977) who found only one isoenzyme in all tissues. It was desirable to select an enzyme with at least two, and preferably three, isoenzymes in order to increase the likelihood of detecting tissue-specific isoenzyme patterns therefore glutamate dehydrogenase was rejected on this basis.

Arylamidase (L-leucyl-peptide hydrolase, E.C.3.4.1.1), formerly known as leucine aminopeptidase, hydrolyses L-leucine-β-naphthylamide and has a wide tissue distribution with distinctive patterns of up to four isoenzymes in human tissues (Smith and Rutenburg, 1966). Multiple molecular forms have also been reported in the bovine (Teleha and Slesarova, 1976). This enzyme was not investigated because its substrate, L-leucine-β-naphthylamide produces the carcinogen β-naphthylamide on hydrolysis.

Gamma-glutamyl transpeptidase [(ɣ-glutamyl)-peptide: amino acid ɣ glutamyltransferase, E.C.2.3.2.2], which catalyses the initial reaction in the degradative metabolism of glutathione, exists as multiple forms in human serum and tissues, but opinion is divided on the number of isoenzymes present (Patel and O'Gorman, 1973;

Fig. 1.1. The glycolytic pathway (after Wilkinson, 1970)



Azzopardi and Jayle, 1973). The enzyme has a wide tissue distribution with the highest levels being found in the liver, kidney and pancreas. Several authors have attempted to correlate serum isoenzyme patterns with pancreatic and hepatobiliary disease in man, but results have been equivocal. Patel and O'Gorman (1973) reported the appearance of additional bands in the serum of patients with hepatic disease, and myocardial infarction, while Azzopardi and Jayle (1973) considered serum gamma-glutamyltranspeptidase isoenzymes to have no diagnostic value in hepatobiliary disease in man. The multiple forms of this enzyme are now thought to be due to aggregation with lipoproteins (Freise, Schmidt and Magerstedt, 1976; Huseby, 1978) and are therefore not true isoenzymes. This enzyme was not considered as suitable for inclusion in this work because of this controversy surrounding the nature of its multiple forms.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1), has a low substrate specificity and can act either as a hydrolase or as a phosphotransferase (Fishman, 1974). It is known to be involved in calcification and possibly in transport of phosphate across cell membranes and absorption of calcium across the intestinal brush border (Warnes, 1972), but its exact functions are still uncertain. Up to four isoenzymes are found in normal human serum,

originating from liver, bone, intestine and, in pregnant women, from placenta.

Alkaline phosphatase isoenzyme studies are of value in the diagnosis of hepatobiliary and bone disorders in man (Lee and Kenny, 1975; Warnes, Hine and Kay, 1977; Burlina and Bugiardini, 1978) but, as mentioned in the Introduction, the normal serum alkaline phosphatase levels are very variable in ruminants (Allcroft and Folley, 1941) while in sheep the "intestinal" isoenzyme varies with blood group (Healy, 1974; Healy, 1975b; Healy and Davis, 1975) and diet (Healy and Davis, 1975). For these reasons the estimation of alkaline phosphatase isoenzymes in sheep was considered to be of questionable value in the detection of specific tissue damage.

Lactate dehydrogenase (L-lactate:NAD oxidoreductase, E.C.1.1.1.27) catalyses the final reaction in the glycolytic pathway under anaerobic conditions.

In aerobic conditions, one molecule of glucose is converted by a series of ten reactions (Fig. 1.1) to two molecules of pyruvic acid with the net production of energy in the form of the high-energy bonds of two molecules of adenosine triphosphate (ATP). The pyruvate thus formed, enters the tricarboxylic acid cycle. However, when the amount of oxygen is limiting, for example in rapidly contracting skeletal muscle, pyruvate is reduced to lactate:

Pyruvate + NADH + H^+ $\xrightarrow{\text{lactate dehydrogenase}}$ L-lactate + NAD^+

The regeneration of NAD^+ by this means, allows glycolysis to proceed under anaerobic conditions since NAD^+ is required for the oxidation of glyceraldehyde-3-phosphate, in the sixth step of this pathway. The lactate formed diffuses into the circulation and is oxidised to pyruvate in the liver, followed by the re-formation of glucose from pyruvate via the gluconeogenic pathway in the liver.

Lactate dehydrogenase has a wide tissue distribution and occurs as five isoenzymes, each of which is detectable in most tissues.

A distinct isoenzyme pattern occurs in the tissues in many species (see Chapter 5). As previously mentioned the estimation of serum lactate dehydrogenase isoenzymes is a well established diagnostic aid in human medicine and has also been used, to a lesser extent, in the detection of liver and muscle damage in several domestic species, including sheep. Lactate dehydrogenase was chosen for further study for the following reasons:

(a) it has a wide distribution but each tissue shows an almost tissue-specific pattern.

(b) the value of lactate dehydrogenase isoenzyme estimations has not been fully investigated in the detection of damage to tissue other than muscle and liver, in sheep.

Fructosediphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, E.C.4.1.

2.13) is a cytoplasmic enzyme catalysing the following reaction, which constitutes the fourth step in the glycolytic pathway (see Fig. 1.1):

fructose-1,6-diphosphate $\xrightarrow{\text{aldolase}}$ dihydroxy-acetone phosphate + glyceraldehyde-3-phosphate

Aldolase exists as three "parent" isoenzymes, A, B and C (Penhoet et al., 1967) with the formation of additional "hybrid" isoenzymes by recombination of the subunits of isoenzymes A and B, or A and C, in vivo (Penhoet, Rajkumar and Rutter, 1966). Aldolase was considered to be suitable for further investigation because although it has a wide tissue distribution, the isoenzyme pattern varies between tissues in man (Arnstall, Lapp and Trujillo, 1966; Lebherz and Rutter, 1969) and in the domestic animals including sheep (Sheedy and Masters, 1969). There are few reports of the diagnostic use of serum aldolase isoenzymes in man (Lang and Würzburg, 1974; Graubaum and Wagenknecht, 1975) and none in the domestic animals.

Glucosephosphate isomerase (D-Glucose-6-phosphate ketol-isomerase, E.C.5.3.1.9), also known as phosphohexose isomerase or phosphoglucose isomerase, is a cytoplasmic enzyme catalysing the second reaction in the glycolytic pathway (see Fig. 1.1):

glucose-6-phosphate $\xrightarrow{\text{glucosephosphate isomerase}}$ fructose-6-phosphate

Like the other glycolytic enzymes glucosephosphate isomerase has a wide tissue distribution. Much

controversy exists as to whether the multiple forms of this enzyme are true isoenzymes, or artifacts (Yoshida and Carter, 1969; Blackburn et al., 1972; Mo, Young and Gracy, 1975). At least in man, the isoenzyme pattern appears to be similar in different tissues (Detter et al., 1968) indicating that serum isoenzyme studies would be unlikely to provide any information as to the site of tissue damage.

Genetically determined variants of glucose-phosphate isomerase are known to occur in man and in domestic animals including rabbits, pigs and horses. No reports have been published describing genetic variants of this enzyme in sheep, and it was of interest to determine whether such variants exist in this species, and if so, whether lambs with different variants show differential growth rates, as has been demonstrated for the NADP – dependant dehydrogenases (Baker and Manwell, 1977).

Creatine kinase (ATP: creatine phosphotransferase, E.C.2.7.3.2) catalyses the reversible phosphorylation of creatine by adenosine triphosphate.

phosphocreatine + ADP $\xrightleftharpoons{\text{creatine kinase}}$ creatine + ATP

Phosphocreatine forms a reservoir of high-potential phosphoryl groups. In actively contracting muscle and to a lesser extent in other tissues, creatine kinase catalyses the transfer of a phosphoryl

group from phosphocreatine to ADP with the formation of ATP. The latter is required in the first and third reactions of the glycolytic pathway (Fig. 1.1). The formation of ATP from phosphocreatine + ADP is necessary since the phosphoryl group cannot be transferred directly from phosphocreatine to glucose.

Creatine kinase is often considered to be a skeletal, heart and smooth muscle-specific enzyme but has, in fact, a wide tissue distribution (Tsung, 1976) with substantial amounts being present in the brain and digestive tract. Studies on the three isoenzymes of creatine kinase in serum are mainly confined to the detection of damage to skeletal or heart muscle in man (Rosalki, 1965; Sherwin, Siber and Elhilali, 1967). Since the work to be described in this thesis was started, creatine kinase isoenzymes have also been studied in the serum of dogs (Knob and Seidl, 1980), pigs (Bickhardt and Schwabenbauer, 1981) and horses (Johnson and Perce, 1981), but neither the serum and tissue distribution nor the diagnostic value of creatine kinase isoenzymes have been studied in the sheep. It was therefore of considerable interest to investigate these parameters, particularly with reference to the detection of damage to tissues other than muscle.

Aspartate aminotransferase (L-Aspartate: 2-oxoglutarate aminotransferase, E.C.2.6.1.1) formerly

known as glutamate-oxaloacetate transaminase, catalyses the following reaction, involving the co-enzyme pyridoxal phosphate:

L-aspartate + ∝-oxoglutarate $\xrightarrow{\text{aspartate aminotransferase}}$ oxaloacetate + L-glutamate.

In general the initial step in the degradation of surplus amino acids is the removal of their ∝-amino groups by transamination to an ∝-keto acid (oxaloacetate). The ∝-amino groups combine with ∝-oxoglutarate to form glutamate which is oxidatively deaminated to give NH_4^+ and ∝-oxoglutarate. NH_4^+ is then converted to urea via the urea cycle. Aspartate aminotransferase exists in tissues as a mitochondrial isoenzyme and as a varying number of cytoplasmic isoenzymes depending on the species and method of isoenzyme separation. Both mitochondrial and cytoplasmic isoenzymes are present in all tissues except mature red blood cells in which only the cytoplasmic isoenzyme occurs. While the isoenzyme patterns are similar in different tissues, Schwartz and Bodansky (1966) noticed that the mitochondrial isoenzyme only appeared in human serum during the acute phase of several diseases. So, although it would provide little or no information regarding the site of tissue damage, the estimation of serum aspartate aminotransferase isoenzymes might be of use in determining the severity of the damage since more severe cellular

damage would be necessary to disrupt the mitochondria than to cause leakage of the cytoplasmic isoenzymes. The appearance of the mitochondrial isoenzyme in serum has also been observed in acute liver necrosis in the rat (Gabrielli and Orfanos, 1968) but apparently not in other species. This enzyme was therefore selected in order to investigate its value as an indicator of the severity of cellular disruption in the sheep.

Thus, of ten enzymes considered for inclusion in this study the five selected for investigation were lactate dehydrogenase, aldolase, glucosephosphate isomerase, creatine kinase and aspartate aminotransferase.

CHAPTER 2

MULTIPLE FORMS OF ENZYMES - REVIEW OF THE LITERATURE

The presence of substances in gastric juice which were involved in digestion was first recognised in 1783 when Spallanzani fed hawks on meat and trained the birds to vomit at various intervals after feeding to enable him to observe the progressive liquefaction of the meat. However, it was not until the following century that Schwann (1836) and Kühne (1867) demonstrated the presence of these substances which they called pepsin and trypsin, respectively, in the gastrointestinal tract.

In 1825, Berzelius had found small amounts of substances which accelerated chemical reactions but were not changed by the reaction. He proposed that the chemical activities in living tissues depended on the existence of these "catalysts" in cells. More than seventy years later, the Buchner brothers (Buchner, 1897) demonstrated that yeast extract, free of living cells, was capable of producing alcohol from sugar. Because of the role of yeast in their discovery, the catalysts of living cells were called "enzymes" meaning "in yeast".

Following the first preparation of an enzyme in crystalline form in 1926 when Sumner crystallised urease, the existence of many other enzymes was soon recognised. Barron and Hastings (1933) isolated

an enzyme catalysing the formation of pyruvic acid from lactic acid, which they called " α-hydroxyoxidase", later to become lactate dehydrogenase. In the same year, Lohmann described the enzymatic activity of glucosephosphate isomerase. In 1934, Meyerhof and Lohmann studied the reaction involving the enzyme which they called "zymohexase", now called fructose-diphosphate aldolase. Braunshtein and Kritzmann (1937) discovered the process of transamination in the reversible formation of aspartate from glutamate and Cohn (1942) showed that "transaminase", later named aspartate aminotransferase catalysed the reaction, while in 1943, Benga first isolated creatine kinase. These enzymes were assumed to be single entities and it was not until 1943 that this was brought into question.

Warburg and Christian (1943) found that aldolase from yeast differed from aldolase from animal tissues while other investigators reported the existence of pepsin, chymotrypsin, trypsin, xanthine dehydrogenase and lysozyme in a number of different forms in different species (cited by Wilkinson, 1970).

Differences between the same enzymes in the same tissues of different species have also been demonstrated, for example, Paul and Fottrell (1961) found species differences in the properties of esterase, alkaline phosphatase, peroxidase and catalase.

Enzymes catalysing a similar reaction but differing in other properties may occur in different tissues of the same species, (Hers and Kusaka, 1953; Wieland and Pfleiderer, 1957) and at one time it was suggested that these forms might be organ-specific (Warburg, 1948). In 1950, Meister observed that crystalline bovine heart lactate dehydrogenase consisted of two components with different electrophoretic mobilities. Although Meister considered the lactate dehydrogenase activity to be associated with one component, Neilands (1952) demonstrated enzyme activity in both fractions. Other examples of enzyme heterogeneity in the same tissue were soon reported and Junger and Gunnar (1957) observed two or three electrophoretic components of aspartate aminotransferase depending on the conditions of electrophoresis.

The classification of multiple forms of enzymes such as these is complex. In 1959, Markert and Møller proposed the term "isozyme" to describe different proteins with similar catalytic activity, "isoenzyme" now being the accepted alternative (IUPAC-IUB Commission on Biochemical Nomenclature, 1976). The definitions recommended by the Commission on Biochemical Nomenclature are as follows:

(a) Multiple forms of the enzyme - a broad term covering all proteins catalysing the same reaction and

occurring naturally in a single species.

(b) Isoenzymes - multiple forms of enzymes arising from genetically determined differences in primary structure.

(c) Allelozymes - genetically variant enzymes (included under the heading "isoenzymes").

(d) Interconvertible enzymes - enzymes existing in at least two well defined, reversibly convertible forms, produced by covalent modifications of amino acid side chains under biological conditions.

THE CHEMICAL NATURE OF ENZYME HETEROGENEITY

The nature of enzyme heterogeneity will be discussed under two headings - isoenzymes and their sub-forms and genetic variants of isoenzymes (allelozymes).

ISOENZYMES AND THEIR SUB-FORMS.

In general, the structure of isoenzymes is determined by two or more gene loci coding for similar, but structurally distinct polypeptide chains (Harris and Hopkinson, 1976). This phenomenon is often attributed to the occurrence of gene duplication during evolution. The products of different gene loci may be present in the same cell but may show differences in their relative rate of synthesis in different tissues or in the same tissue at different times during pre- or post-natal development. Multiple loci are responsible for the formation of different types of subunit in the

same enzyme, for example Cahn et al. (1962) demonstrated that lactate dehydrogenase was a tetramer composed of two subunit types which they named 'M' (from skeletal muscle) and 'H' (from heart). They proposed that the subunits were probably coded by different genes and combined to form five isoenzymes of lactate dehydrogenase - HHHH, HHHM, HHMM, HMMM and MMMM (designated LDH_1, LDH_2, LDH_3, LDH_4 and LDH_5 respectively), the isoenzymes with two different subunits being called "hybrids" of the two parent forms. A sixth isoenzyme, LDHX, has been demonstrated in testicular tissue, and is considered to be the product of a third locus.

Many enzymes are now known to exist as different subunit combinations. Penhoet et al. (1967) showed that fructose diphosphate aldolase was a tetramer with three parent forms, A, B and C, each consisting of four similar subunits to give AAAA, BBBB and CCCC. Thus, five-member sets may form in an identical manner to that of lactate dehydrogenase by hybrid formation from two parent types to give AAAA, AAAB, AABB etc. However, only A-B and A-C hybrid sets have been demonstrated in vivo (Penhoet, Rajkumar and Rutter, 1966). Similarly, trimers would be expected to have four different combinations of two subunit types and dimers such as creatine kinase (Eppenberger, Dawson and Kaplan, 1967) to have three combinations.

These theories are supported by amino acid determinations in different parent and hybrid forms and by demonstration of gradual changes in the physical and chemical properties such as electrophoretic mobility (Markert, 1963) and heat stability (Tollersrud, 1970) in hybrid sets. Further evidence is provided by immunological studies and the hybrid forms of lactate dehydrogenase, creatine kinase and aldolase have been shown to cross-react with antisera raised against the parent forms consisting of one subunit type (Cahn et al., 1962; Eppenberger, Dawson and Kaplan, 1967; Matsushima et al., 1968). Formation of hybrid forms in vitro from parent types has provided conclusive evidence of subunit structure (Markert, 1963; Penhoet et al., 1967).

Some enzymes whose subunit structure is well known show additional forms, for example creatine kinase which has three possible subunit combinations exists as a fourth isoenzyme in the mitochondria (Jacobs, Heldt and Klingenberg, 1964). This isoenzyme is biochemically and immunologically distinct from the cytoplasmic forms (Kwong and Arvan, 1981) but has yet to be purified although it is known to be a dimer. A great deal of controversy exists regarding the presence or absence of isoenzymes of glucosephosphate isomerase. Three main isoenzymes have been described (Detter et al., 1968; Tsuboi and Fukunaga, 1971).

Yoshida and Carter (1969) considered that one of the "isoenzymes" of crystalline rabbit muscle glucose-phosphate isomerase was derived from one of the other two *in vitro*. They found five bands in tissue extracts which they attribute to oxidation of disulphide bonds of the two "true" isoenzymes. However, Blackburn *et al*. (1972) disagreed with Yoshida and Carter, stating that all three forms which they demonstrated in crystalline rabbit muscle glucose-phosphate isomerase or crude muscle extracts represented different states of the same protein which could be interconverted by treatment with a reducing agent and by exposure to oxidative conditions. In fact, the position regarding the presence or absence of glucosephosphate isomerase isoenzymes is complicated by the existence of genetic variants in many species and Mo, Young and Gracy (1975) have adopted an intermediate view, stating that multiple forms observed in homozygous individuals only, are the result of oxidation of sulphydryl groups *in vitro*.

Other isoenzymes such as the cytoplasmic and mitochondrial isoenzymes of aspartate aminotransferase each consist of two similar polypeptide chains which differ between isoenzymes in amino acid composition (Martinez-Carrion and Tiemeier, 1967) and immunological properties (Campos-Cavieres and Munn, 1973). A hybrid form has not been reported, probably because

of the existence of the two isoenzymes in different parts of the same cell (Edwards, Hopkinson and Harris, 1978). However, several workers have demonstrated that the cytoplasmic and mitochondrial isoenzymes are not electrophoretically homogeneous. Boyde and Latner (1962) reported two anodally migrating isoenzymes in pathological human serum while Block, Carmichael and Jackson (1964) reported three anodally migrating and two cathodally migrating bands in normal human serum. Gabrielli and Orfanos (1968) separated aspartate aminotransferase from rat serum and liver into five distinct bands, two which migrated anodally and three which migrated cathodally. The former appeared to be the cytoplasmic and the latter the mitochondrial form. The nature of these sub-forms is still uncertain.

Subforms of many enzymes have been demonstrated by isoelectric focusing. In isoelectric focusing, proteins are separated in a pH gradient according to their isoelectric point.

The reasons for the existence of sub-bands when isoenzymes are separated by isoelectric focusing have never been fully explained. The major sources of this heterogeneity may be classified as follows (after Righetti and Drysdale, 1976):

1. Primary structure.

2. Variations in prosthetic groups or in carbohydrate, lipid or nucleic acid content.

3. Ligand binding e.g. substrates and cofactors.

4. Heterogeneity in bound ligands, differences in metal content or redox state of metalloenzymes.

5. The existence of different conformations ("conformers") of the same molecular structure, which can be interconverted by rotation about bonds.

6. Post-synthetic modification in vivo or in sample preparation e.g. instability of molecules at their isoelectric point, interaction with the "carrier ampholytes" which produce the pH gradient, oxidation or reduction of samples with isoelectric points too close to the electrodes and denaturation or precipitation during focusing.

Since Dale and Latner (1968) observed that lactate dehydrogenase was resolved into more than five bands on isoelectric focusing, many authors have attempted to provide explanations. The most complete account has been given by Klose and Spielmann (1975) who showed that in addition to the five isoenzymes usually observed, two further sets of five bands are demonstrable in mouse organs. One set was found in all organs and was considered to be due to the existence of two types of M subunit which generated five bands by recombination (i.e. $M^1M^1M^1M^1$, $M^1M^1M^1M^2$, $M^1M^1M^2M^2$, $M^1M^2M^2M^2$ and $M^2M^2M^2M^2$). The other set of five was found only in testis and was the result of two different types of X subunit. LDH_1 (HHHH) formed

only one band on isoelectric focusing. They observed several additional bands which appeared either regularly or less consistently. Since the H subunits seemed to combine mainly with one type of M subunit, the authors considered that some of these weak, additional bands could have been due to combination of H subunits with the second type of M subunit. The existence of artefactual bands was to some extent ruled out for the following reasons:

(a) Tetramers of the two types of M subunit were also demonstrable by chromatography.

(b) Multiple bands were observed with purified LDH_5 (MMMM) which could not have been caused by complexing with tissue proteins during focusing.

(c) Isoelectric focusing in two dimensions (see Chapter 3) did not produce more bands than the one-dimensional technique.

(d) The presence of increased amounts of carrier ampholytes did not alter the band pattern indicating that complexing with ampholytes was unlikely.

(e) Site of sample application in the pH gradient did not alter the band pattern i.e. migration through either the anodic or cathodic part of the gradient did not affect the band pattern.

A similar situation appears to pertain for aldolase. Susor, Kochman and Rutter (1969) resolved crystalline aldolase A into five components by

isoelectric focusing and proposed that this five-member set was due to the presence of two different type A subunits while Gürtler and Leuthardt (1970) demonstrated the same phenomenon for aldolase B. The two types of aldolase A subunit, α and α' have been isolated, treated with trypsin to split them into peptides and subjected to two-dimensional chromatography to produce "peptide maps" (Susor, Kochman and Rutter, 1973) which differed only slightly between the two subunit types. Other explanations for type A subunit differences which they suggested were the presence of covalently bound phosphate, carbohydrate and sialic acid residues, or deamidation and proteolysis *in vivo* or during purification. The most likely explanation appeared to be differences in the third amino acid from the C-terminal end of each subunit molecule, type α having an asparagine residue and type α' an aspartic residue.

Purified creatine kinase from rabbit muscle (MM isoenzyme) has been shown to consist of two different M subunit types to give three bands on isoelectric focusing - two homodimers and one heterodimer (Cattan *et al.*, 1978). Chapelle and Heusghem (1980) resolved human serum creatine kinase from patients with myocardial infarction into three MM isoenzyme bands and two MB isoenzyme bands by electrophoresis, while two additional MM bands were found

after isoelectric focusing. In tissues however, only two MM and one MB band were observed after focusing. They concluded that the multiple forms in serum were due to post-synthetic modification by a thermolabile factor present in serum. The appearance of the variants in serum was correlated with time after myocardial infarction. Similar results were obtained by Yasmineh, Yamada and Cohn (1981) who also demonstrated that the variants were present, albeit to a lesser extent, in normal human serum. Rosenberg, Eppenberger and Perriard (1981) have shown that M and B subunits of chicken, rat and mouse tissues each show two forms on electrophoresis which they proposed were the products of different genes.

Campos-Cavieres and Munn (1973) resolved the purified cytoplasmic isoenzyme of sheep liver aspartate aminotransferase into three pairs of bands by isoelectric focusing. They considered that the nature of the multiple forms was probably similar to that of the pig heart subforms which have identical amino-acid compositions. One hypothesis is that they are conformers with a different environment of charged groups, leading to a different net surface charge (Banks et al., 1968).

Whatever the reason for their existence, sub-forms cannot be classified as true isoenzymes until they are

shown to be due to genetically determined differences in primary structure and must therefore come under the broad heading of "multiple forms".

ALLELOZYMES

At any one gene locus, a number of different alleles may occur coding for a distinct version of a polypeptide chain. Thus, the primary structures of isoenzymes may vary between individuals of the same species according to the alleles at the particular locus. Heterozygous individuals will show a more complex pattern than homozygous individuals. If the isoenzyme consists of one polypeptide chain, heterozygotes will show a simple mixture of each of the forms seen separately in the homozygotes, but if the isoenzymes contain more than one polypeptide chain, "hybrid" isoenzymes not present in either homozygote will be observed (Harris and Hopkinson, 1976).

Alleles coding for variants of a polypeptide usually induce substitution of a neutral by a basic or acidic amino acid or vice versa or substitution of a basic for an acidic amino acid or vice versa. If this produces an alteration in charge, the variant isoenzymes will be distinguishable by electrophoresis.

If isoenzymes variants arise frequently enough for individuals in a population to be classified into two or more groups, this is referred to as "enzyme

polymorphism".

In man, over seventy enzymes have been shown to exist as allelozymes. Glucosephosphate isomerase, which is the most extensively studied polymorphic enzyme in man, was first reported to show genetic variants in 1968. Fitch, Parr and Welch (1968) demonstrated five variants in haemolysates each with three isoenzymes of differing electrophoretic mobility, whereas Detter et al. (1968) found ten variants each with 3-9 isoenzymes, eight of which had three main components. VandeBerg and Stone (1978) in a study of human and Rhesus monkey glucosephosphate isomerase considered that the phenotypes of the enzyme were controlled by codominant autosomal alleles i.e. all alleles involved were fully expressed in the heterozygote. They observed two different three-band patterns in homozygous individuals, and a six-band pattern in heterozygotes. Gibson et al. (1978), reported that the isoenzymes containing two identical subunits (homodimers) formed one band on electrophoresis while heterodimers formed three bands. One of the human variants studied by Gibson et al. was more stable in the presence of guanidium hydrochloride and proteases than the other variants. Satoh and Mohrenweiser (1979) exploited differences in thermostability and

stability in the presence of urea to study previously undetected allelozymes which were not distinguishable by electrophoresis. They were able to detect amino-acid substitutions which altered the conformation of the molecule but did not affect its charge or kinetic properties, the latter indicating that the structural alteration did not affect the active site of the enzyme.

In laboratory animals, allelozymes of glucose-phosphate isomerase have been described in the mouse (Carter and Parr, 1967; Charles and Lee, 1980), and rabbit (Welch, Fitch and Parr, 1970), while Dando (1974) reported variants in many species of fish.

In the domesticated animals, glucosephosphate isomerase variants have been recognised in several species. In the horse, Sandberg (1973) observed four variants in two Swedish breeds. By mating horses with specific variants and noting the distribution of the variant types in the offspring, Sandberg concluded, like VandeBerg and Stone (1978), that the variants were controlled by codominant autosomal alleles, in this case by three alleles.

In the pig, three variants have been reported (Jørgensen et al., 1976), one of which appeared to be associated with adverse reactions to the volatile anaesthetic, halothane. The domestic cat has been

reported to show three complex isoenzyme patterns controlled by two codominant autosomal alleles, and three additional variant forms (Auer and Bell, 1980).

Genetic variants of this enzyme are known to exist in the sheep (Yoshida and Carter, 1969), but their results have not been published. Apparently there is only one published report of the genetic variation of this enzyme in the sheep - Baker and Manwell (1977) reported that it was not polymorphic. These authors investigated genetic variation of 21 enzymes in sheep and found polymorphism in only four - "malic enzyme", isocitrate dehydrogenase, catalase and esterase.

THE PHYSIOLOGICAL SIGNIFICANCE OF ISOENZYMES

In many cases, isoenzymes are found in tissues in proportions which suggest that they are not formed by a random combination of their subunits. Moreover, differences in the proportion of isoenzymes in different organs suggests a physiological basis for their existence.

Allen (1961) demonstrated that lactate dehydrogenase isoenzymes from mouse tissues varied in their substrate specificity and in their behaviour during physiological changes. He observed that in lactating mammary gland the electrophoretically fastest migrating isoenzyme (LDH_1) showed the highest activity whereas in non-lactating mammary gland, the slowest-

migrating isoenzyme (LDH_5) predominated and concluded that these differential responses were probably important in terms of cellular function.

Cahn et al. (1962) suggested that the H and M subunits of lactate dehydrogenase have different functions. LDH_4 and $_5$ which contain mainly M subunits, permit rapid accumulation of lactate and are therefore found in tissues such as skeletal muscle, where anaerobic glycolysis predominates, whereas LDH_1 and $_2$ which contain mainly H subunits are found in tissues such as heart and brain where pyruvate is oxidised via the tricarboxylic acid cycle. Aldolase isoenzymes show different substrate specificities suggesting different metabolic roles. Aldolases A and B show a fructose diphosphate: fructose-1-phosphate activity ratio of about 50:1 and 1:1 respectively (Penhoet et al., 1966) while aldolase C has a ratio of about 7.5:1 (Hatzfeld et al., 1977). Aldolase A predominates in tissues with an active glycolytic pathway such as skeletal muscle, and in rapidly growing cells, whereas aldolase B occurs in cells with a greater metabolic diversification (Rutter et al., 1963).

The fact that the same tissue may show a species-specific isoenzyme pattern has also been shown to reflect differences in the predominant metabolic pathways. Fine, Kaplan and Kuftinec (1963) showed

that ruminant liver lactate dehydrogenase consisted of isoenzymes with a higher proportion of H subunits than the liver of other species. The authors proposed that oxidative metabolism was of greater importance in the liver of ruminants (i.e. the formation of pyruvate from lactate for incorporation into the tricarboxylic acid cycle) and that pyruvate is an intermediate in the formation of glucose from the volatile fatty acids produced in the rumen.

Similarly, changes may occur in the isoenzyme pattern of the same tissue in an individual animal. Hinks and Masters (1964) found that ruminant liver and heart lactate dehydrogenase showed an increasing proportion of H subunits with increasing gestational age, whereas skeletal muscle showed an increasing proportion of M subunits. Davis et al. (1973) in a study of the developing bovine foetus reported an increasing proportion of H subunits with age in skeletal muscle, brain, thyroid, pancreas, lung and heart. The results for skeletal muscle conflict with those of Hinks and Masters.

After birth, changes in the proportions of isoenzymes tend to reflect a continuation of pre-natal changes. Thus neonatal mouse heart lactate dehydrogenase shows an increasing proportion of LDH_1 which contains only H subunits and a decreasing proportion of LDH_5 containing only M subunits (Courtney and Ebron, 1978). Walden and Schiller (1980) reported

that foetal hamster small intestine showed low percentages of LDH_1 and LDH_2 with increasing levels after birth, suggesting an increased oxidation of pyruvate by rapidly growing tissue since the presence of more H subunits would favour the formation of pyruvate from lactate.

Creatine kinase in chicken and rabbit skeletal muscle changes during ontogeny from a predominance of the BB isoenzyme to the MM isoenzyme which is present in the highest concentration in adult muscle (Schapira et al., 1968). All three creatine kinase isoenzymes are present in neonatal mouse hearts, but by 18 days old the BB isoenzyme is no longer present. However, 6 days after birth, an isoenzyme which is specific to mitochondria appears (Hall and DeLuca, 1975). The authors suggested that the mitochondrial isoenzyme catalyses the formation of phosphocreatine + ADP from creatine + ATP. In foetal and neonatal mouse heart, ATP is needed for the synthesis of new cellular constituents therefore the mitochondrial isoenzyme is absent since it would not be advantageous to produce phosphocreatine at the expense of ATP. After the neonatal stage, this isoenzyme appears and synthesises more phosphocreatine relative to ATP.

In foetal rat heart and skeletal muscle, enolase isoenzyme $\alpha\alpha$ is found in the highest concentration, but is partially replaced by isoenzymes $\alpha\beta$

and ββ in adult heart and completely by isoenzyme ββ in adult skeletal muscle (Rider and Taylor, 1975). They concluded that since a number of glycolytic enzymes including enolase bind to muscle proteins, the functional difference between the isoenzymes might lie not in variation in their kinetic properties but in their differential affinity for specific components of particular tissues. However, they had no evidence to support this hypothesis. Similar changes in enolase isoenzymes have been reported in the mouse (Fletcher et al., 1978).

Schapira et al. (1968) found almost pure aldolase C in chicken embryo skeletal muscle, with evidence of A-C hybrid forms, whereas adult muscle contains almost pure aldolase A.

In human embryos, the aldolase pattern in kidney and small intestine changes from a predominance of aldolase A to a predominance of aldolase B (Rehbein-Thöner and Pfleiderer, 1977). Since glucose is the main energy source of foetal metabolism, aldolase A would be expected to predominate since its high activity for fructose diphosphate cleavage favours glycolysis. In contrast, aldolases B and C favour gluconeogenesis.

Kawachi et al. (1973) reported that the gene for aldolase B is expressed to a greater extent than that of aldolase A in the mucosa of the whole digestive tract in the neonatal rat. However, during weaning

aldolase A subunits (i.e. the isoenzymes of the five-membered set derived from hybridisation of the subunits of aldolase A and B, which contained mainly A subunits) predominated in the stomach and colon whereas in the small intestine, both aldolases A and B increased during weaning and decreased again after weaning. The existence of hybrid isoenzymes containing both A and B subunits indicates that the genes for both subunits may function in the same cell in intestinal mucosa. The increases observed in aldolase A and B in the small intestine were thought to be related to morphological changes associated with weaning.

The role of isoenzymes in metabolism has been discussed by Ureta (1978). He stated that the reason for the existence of isoenzymes may be explained by their showing differences in one or more of the following:

(a) affinity for substrates or cofactors

(b) substrate or cofactor specificities

(c) response to allosteric effectors (i.e. response to small molecules which interact with protein molecules leading to changes in the shape of the protein and a consequent alteration of the interaction of that protein with a third molecule)

(d) subcellular localisation

(e) susceptibility to dietary and/or hormonal treatments

(f) time of appearance during differentiation

Several metabolic pathways are known to be organised as "multienzyme complexes" allowing efficient transformation of the substrate, the enzymes being bound together so that the product of one reaction becomes the substrate of the next without loss of the intermediate products formed. Ureta presented a hypothesis that metabolic pathways are unidirectional chain reactions catalysed by specific isoenzymes associated as "polyisoenzymic complexes". Thus sub-pathways within a metabolic pathway (for example, the forward and backward direction of a series of reactions would constitute two sub-pathways) are each catalysed by polyisoenzymic complexes containing different isoenzymes. He considered that differences in net charge distributed over the surface of an isoenzyme molecule would determine with which isoenzymes of the adjacent enzymes in the pathway it would associate to form the polyisoenzymic complex. The actual amount of each isoenzyme in a cell would depend on the number of times each different complex is represented in the cell which in turn would depend on the type of metabolism. This would result in quantitative differences in isoenzyme levels between tissues. Although Ureta's hypothesis has not been proven, a certain amount of indirect evidence exists to confirm his proposals.

The physiological significance of genetic variants of isoenzymes is also uncertain. It is usually assumed that mutant forms only persist if they confer some advantage on the individual. In many cases, enzyme polymorphism produces no detectable effect on the individuals concerned (Wilkinson, 1970) while in other instances the effect may be disadvantageous e.g. several "deficiency" alleles of glucosephosphate isomerase have been recognised in man, which cause chronic haemolytic anaemia (Harris and Hopkinson, 1976). Baker and Manwell (1977) who found "malic enzyme", isocitrate dehydrogenase, catalase and esterase to be polymorphic in the sheep, suggested three explanations for the biological significance of NADP-dependant dehydrogenases (i.e. "malic enzyme", isocitrate dehydrogenase and catalase) and their polymorphism. Firstly, all three of these enzymes have hybrid zones in the heterozygote and in the case of "malic enzyme" most of the activity is present in these zones. The authors suggested that the hybrid isoenzymes might have a faster rate of action or a different degree of feedback regulation. Secondly, animals with different genotypes showed differences in growth rate. Thirdly, polymorphism of NADP-dependant dehydrogenases and esterases, which are both involved in detoxication reactions may indicate different capacities of genotypes for detoxication. However, these suggestions,

with the exception of the second, do not appear to have been substantiated.

This uncertainty about the significance of allelozymes appears to be typical of the state of knowledge regarding the reasons for the existence of multiple forms of enzymes in general.

CHAPTER 3

METHODS FOR THE STUDY OF ISOENZYMES - REVIEW OF THE LITERATURE

Procedures for the study of isoenzymes can be divided into non-separative and separative methods.

Non-separative methods involve selective destruction of some isoenzymes by physical, chemical or immunological means, followed by assay of the remaining enzyme activity, or adjustment of the reaction conditions so that certain isoenzymes are preferentially assayed (Rosalki, 1974a).

Separative methods involve the physical separation of isoenzymes followed by one of two procedures:

(a) the isoenzymes are eluted from the separation medium and the activity of each is assayed.

or (b) the isoenzymes are incubated with substrate in situ to form a coloured product or a substance which fluoresces under ultraviolet light. The product is quantified by densitometric or fluorometric scanning respectively, since in certain circumstances, its concentration is proportional to the enzyme activity.

In order to save space, techniques not subsequently used will be discussed very briefly.

NON-SEPARATIVE METHODS

HEAT INACTIVATION

Differential inactivation by heat has been used mainly in the study of alkaline phosphatase isoenzymes,

often in conjunction with other techniques such as electrophoresis and chemical inhibition (Rhone, White and Gidaspow, 1974). However, the method is not applicable to mixtures of isoenzymes (Kaplan, 1972; Mercer, 1974; Hall and DeLuca, 1976) and there may be overlap in the behaviour of isoenzymes (Kaplan, 1972). Accuracy is a problem since rates of inactivation change markedly with small variations in temperature (Lee and Kenny, 1975).

CHEMICAL INHIBITION

Differential inhibition by chemical means is also mainly used for alkaline phosphatase isoenzymes, but does not differentiate between all isoenzymes in a mixture (Rosalki, 1974a; Mercer, 1974) since it cannot be assumed that an inhibitor acts specifically on one isoenzyme (Kaplan, 1972).

SUBSTRATE OR CO-ENZYME VARIATION

Adjustment of the reaction conditions for the preferential assay of certain isoenzymes has been reported for alkaline phosphatase (Dulis and Wilson, 1978), lactate dehydrogenase (Freedland, Theis and Cornelius, 1963) and aldolase (Schapira, 1961) but none of these authors attempted to quantify the isoenzymes. Kaplan (1972) and Rosalki (1974a) considered it to be unsuitable for mixtures of isoenzymes, while Fritz et al. (1970) found the method to be suitable only for determination of the percentage of the

different subunits of which the isoenzymes are composed and to be less reproducible than electrophoresis or chromatography.

IMMUNOPRECIPITATION

Immunoprecipitation of isoenzymes by specific antisera has its main diagnostic application in the study of creatine kinase isoenzymes. However, most methods are not isoenzyme-specific, and those which are specific are not necessarily quantitative (Usategui-Gomez et al., 1981; Wicks et al., 1982), and only one isoenzyme can be quantified at a time.

SEPARATIVE METHODS

RADIOIMMUNOASSAY

First described by Yalow and Berson (1960) for insulin, radioimmunoassays have been adapted for the quantitation of isoenzymes but are said to have few advantages over electrophoresis. Only one isoenzyme can be quantified at a time, quantitation requires computer analysis and it is not isoenzyme-specific (Homburger and Jacob, 1980; Usategui-Gomez et al., 1981; Wicks et al., 1982).

RADIAL IMMUNODIFFUSION AND ELECTROIMMUNODIFFUSION

In radial gel immunodiffusion an isoenzyme-specific antibody is mixed with the gel before gelling and the sample mixture is placed in a central well in the agarose. A precipitation ring forms round the well, whose diameter relates to the isoenzyme concentration.

In electroimmunodiffusion or rocket immuno-electrophoresis (Laurell, 1966), electrophoresis is performed so that peak-shaped precipitin zones originating from the wells develop and these are stained for enzyme activity. The distance travelled by a precipitate is proportional to the antigen concentration therefore the isoenzyme in the sample can be quantified by comparison with the distance travelled by a standard (Ouchterlony and Nilsson, 1973). However, Clausen (1969) stated that enzyme activity is sometimes reduced or absent after precipitation by antibody.

Radioimmunoassay, radial immunodiffusion and electroimmunodiffusion all have the disadvantage that the production of specific antibody is required, and only one isoenzyme can be quantified at a time (Clausen, 1969) while, with the exception of radio-immunoassay, the isoenzymes under investigation must be immunologically distinct.

ION-EXCHANGE CHROMATOGRAPHY

In 1956, Peterson and Sober introduced the ion-exchange celluloses, carboxymethylcellulose (CM-cellulose) and diethylaminoethylcellulose (DEAE-cellulose) which were suitable for the separation of macromolecules.

When a mixture of ionic solutes comes into contact with the matrix of an ion-exchange bed, the

bed repels ions carrying the same charge as its own functional groups and binds ions of the opposite charge. Strength of binding depends on the size of the charge on the ion, and will change with the pH and ionic strength of the elution buffer (Mikes, 1966). Separation occurs when the buffer flows through the ion-exchange column. The ions undergoing separation are eluted by gradually changing the pH or ionic strength of the buffer flowing through the ion-exchange bed. The eluate is collected in fractions and the substance of interest assayed by conventional means.

Although very sensitive (Mercer and Varat, 1975), assay of many fractions is required (Hall and DeLuca, 1976) and there is a risk of "carry-over" of enzyme into subsequent fractions (Levy and Lum, 1975; Willis, Davies and Baines, 1978; Hamilton et al., 1979). It is rarely used in clinical chemistry because of the existence of simpler alternatives (Rosalki, 1974a).

GEL FILTRATION

The technique of gel filtration was introduced by Porath and Flodin (1959) using Sephadex, a cross-linked dextran. The particles of Sephadex, when allowed to swell in water, form porous beads which act as molecular sieves. When the sample passes down the Sephadex column, molecules larger than the pore size of the beads are rapidly eluted with the

buffer flowing through the column. Smaller molecules enter the bead pores and thus move more slowly through the column, molecules being eluted in order of decreasing molecular size.

The use of gel filtration in enzymology is largely restricted to the estimation of the molecular weights of multiple forms of enzymes such as glucose-phosphate isomerase (Tsuboi and Fukunaga, 1971), and creatine kinase (Kanemitsu, Kawanishi and Mizushima, 1981), and is rarely used in the clinical laboratory because simpler techniques are available (Rosalki, 1974a).

ELECTROPHORESIS

Electrophoresis is the term originally used by Michaelis in 1909 to describe the movement of ions under the influence of an electric field.

The earliest form of the technique, moving boundary electrophoresis, was introduced by Picton and Lindler in 1892. In 1937, Tiselius developed moving boundary electrophoresis for separating proteins and other high molecular weight substances, and suggested that it could provide information about the chemical homogeneity of the material. He (Tiselius, 1957) quantified zones of separated proteins by measuring changes in the refractive index as proteins migrated through the buffer solution.

Although enzyme heterogeneity was first demonstrated using the Tiselius apparatus for crystalline bovine heart lactate dehydrogenase (Meister, 1950), moving boundary electrophoresis has been little used for protein separations due to the difficulties in isolating the separated proteins. With the advent of zone electrophoresis, it has been virtually abandoned.

Zone electrophoresis is a system whereby the solution containing the ions to be separated is supported in a fairly inert material such as paper, starch or agar. The ions remain as zones and can be detected by conventional physical, chemical or biochemical means (Sargent, 1969).

Zone electrophoresis is highly suited to the separation and quantitation of mixtures of proteins including enzymes, since (a) sharp resolution is obtainable, (b) zones can easily be located either by staining <u>in situ</u> or after elution from the supporting medium, (c) the stained zones can be quantified by densitometric or fluorometric scanning and (d) several samples can be analysed simultaneously under exactly the same conditions.

Before considering the different support media, some factors affecting the movement of proteins during zone electrophoresis will be considered.

Factors affecting the movement of proteins during zone electrophoresis

The ability of a protein to migrate in an electric field depends primarily on its net electric charge. The motive force to which both positively (cationic) and negatively charged (anionic) proteins are subjected is equivalent to Q x N, where Q = field strength and N = net charge on the protein. The size and shape of the protein and the type of support medium determine the frictional force which opposes the motive force.

Effects of current and voltage

The passage of a current through a solution is governed by Ohm's law which states $\frac{V \text{ volts}}{R \text{ ohms}}$ = I amps

The rate of migration of ions, which carry the current, can be increased by increasing the current. Ohm's law indicates that the latter may be achieved by either increasing the voltage or decreasing the resistance. Since, in theory, the resistance of the supporting medium is constant, increased current is obtained by raising the voltage. However, as current increases, heat is generated resulting in a decrease in resistance and an increase in the rate of evaporation of solvent in the medium. These two effects tend to oppose each other but the net result on protein migration is unpredictable. The effects of heating can be controlled by either maintaining a constant voltage or a constant current during electrophoresis (Smith, 1976), and by providing means of

cooling the support medium.

Effects of pH, ionic strength and chemical composition of buffer

The net charge on a protein molecule varies with the pH of its environment. Since proteins are "zwitterions", i.e. structures with both positive and negative charges on the same molecule (Taylor, 1973), they can exist in a negatively charged, neutral or positively charged state according to the pH of the buffer solution in the supporting medium. The pH at which positive and negative charges on the protein molecule are balanced is the "isoelectric point" (pI) of the protein which then exists in the form $NH_3^+RCOO^-$. At a pH below the isoelectric point the molecule is positively charged (NH_3^+RCOOH) and will move cathodally, whereas at a pH above the isoelectric point it is negatively charged ($NH_2\ RCOO^-$) and will move anodally. Thus by changing the pH, the same protein can move either anodally or cathodally in an electric field.

The ionic strength of a buffer may be calculated from the equation $\mu = \frac{1}{2} \in mc^2$, where μ = the ionic strength, m = the molarity of an ion, and c = the charge carried by that ion (Sargent, 1969). In general, the higher the ionic strength, the more slowly the proteins undergoing separation will migrate because more of the current is being carried by the buffer ions. In addition, the movement of

protein molecules surrounded by ions of opposite charge is retarded because of mutual attraction (Smith, 1976).

The net effect of increasing buffer ionic strength is lower mobility and increased zone resolution but also increased generation of heat.

The migration of proteins is affected by the chemical composition of the buffer (Ressler, 1973; Ambler and Rodgers, 1980) in the same medium and at the same pH, but the reason for this is not certain. In some cases, interaction between proteins and small molecules in the buffer may occur. The work of Cann (1966) and Cann and Goad (1968), showed that when a single protein interacts reversibly with a small molecule in the buffer, two protein zones may result. This depends on variations in the concentration of the buffer where the protein is migrating. If the ratio of the buffer concentration to protein is sufficiently high, the concentration of the buffer along the migration path of the protein becomes almost constant and the protein migrates as one band.

Electroendosmosis

Most support media for electrophoresis carry ionizable groups such as sulphate and carboxyl groups in agarose and carboxyl groups in paper (Sargent, 1969), which may be ionised at the pH of the electrophoresis buffer. When exposed to an electric

field, these negatively charged groups induce positive charges on surrounding solvent molecules (Kunkel and Tiselius, 1952) causing them to migrate cathodally. This flow of solvent molecules or "electroendosmosis" is in the opposite direction to the migration of most proteins during electrophoresis, and may carry neutral proteins towards the cathode. Electro-endosmosis is generally considered to be undesirable, although it can increase the separation between particular macromolecules such as γ-globulins during electrophoresis and is essential for certain forms of immunoelectrophoresis (Pharmacia, 1978).

Type of support medium

The medium for electrophoresis has a marked effect on the migration of proteins. This will be discussed separately for each medium.

Support Media

Paper

When the existence of multiple forms of enzymes was first recognised in the 1950's, filter paper electrophoresis was already a well established technique for the separation of serum proteins (Kunkel and Tiselius, 1952). Consequently, many of the early isoenzyme separations were carried out on paper.

The tendency of certain proteins to bind to paper rendered early elution techniques unsatisfactory for quantitative estimations (Sargent, 1969). When

proteins are stained *in situ*, paper-protein binding and a high degree of electroendosmosis cause poor resolution of bands and "tailing" (Kunkel and Tiselius, 1952). Hess, in 1958, when separating human serum lactate dehydrogenase isoenzymes, noted that some of the enzyme was inactivated by the paper. Paper has now been superseded by other media for zone electrophoresis.

Agar gel

Agar, an extract of certain red seaweeds, is a mixture of two polysaccharides - neutral agarose and negatively charged agaropectin. The sulphate and carboxyl groups of agaropectin cause both irreversible adsorption of basic substances including proteins and marked electroendosmosis (Hjertén, 1961; Låås, 1972).

Despite its limitations, agar gel has been used successfully for the separation of the isoenzymes of many enzymes including lactate dehydrogenase (Wieme and Van Maercke, 1961; van der Helm, 1962; Gerber, 1966), and creatine kinase (Deul and van Breeman, 1964; Burger, Richterich and Aebi, 1964). However, since the development of a means of separating agarose from agar to produce a superior electrophoresis medium (Hjertén, 1961), agar is not the medium of choice for isoenzyme separations.

Starch gel

Hydrolysed starch was first used for zone

electrophoresis by Smithies in 1955 who was the first to describe the molecular filtration or "sieving" effects of starch gel on proteins which is due to the branching and intertwining carbohydrate chains of which the gel is composed (Gordon, 1969). Thus, proteins are separated not only on the basis of charge, but also on their ability to penetrate the "sieve", i.e. on their size and shape. The concentration of starch can be chosen according to the size of the proteins undergoing separation for optimum resolution. This, combined with the relatively low electroendosmotic properties of starch gel produced superior resolution to that obtained with paper or agar gel.

A vast amount of literature exists on the use of starch gel for electrophoresis of human and animal enzymes. However, the high resolving power of starch should be treated with caution as artefactual bands have been reported due to aggregation or separation of proteins into subunits (Sargent, 1969).

Cellulose acetate

Two forms of cellulose acetate have been developed for electrophoresis, the dry membrane form and the gel form. Both forms consist of cellulose diacetate, the difference being in the presence of chemically-bound water in the gel form, which reduces the rate of evaporation from the gel, and electroendosmosis. Like starch gel, cellulose acetate acts as a molecular sieve.

Kohn (1957), who introduced the medium for serum proteins described it as having minimal protein absorption resulting in the absence of "tailing" and recommended its use for the detection of stained proteins by optical scanning since the membrane, which is white, can be rendered transparent to ultra-violet and visible light by immersion in various oils after electrophoresis. Siede and Seiffert (1977) however, found that rendering the membrane transparent after histochemical staining destroyed both the stain and the membrane itself, whereas Ogunro, Hearse and Shillingford (1977) considered cellulose acetate electrophoresis to be irreproducible for creatine kinase isoenzyme separations.

Agarose gel

The disadvantages of agar gel prompted Hjertén in 1961 to develop a method for separating neutral agarose from negatively charged agaropectin with the aim of producing a medium for zone electrophoresis with low electroendosmotic properties. Hjertén produced agarose gel which had no observable tendency to absorb proteins or low molecular-weight substances. The degree of electroendosmosis was decreased, but not eliminated. Agarose is virtually free of molecular sieving effects so electrophoretic separation depends mainly on charge differences between proteins (Harris and Hopkinson, 1976).

Zone electrophoresis in thin layers of agarose is rapid and produces superior resolution to paper and agar. The gel is transparent and therefore suitable for optical scanning of stained protein zones, is easily handled when cast on a transparent hydrophilic film and can be dried to form a permanent record.

Since its conception in 1961, agarose electrophoresis has become a well-established technique in the medical field. In the late 1970's, commercially prepared agarose plates became available specifically for serum proteins (Corning Agarose Plates, Corning Medical, Palo Alto, California, U.S.A.) or for alkaline phosphatase, creatine kinase and amylase isoenzymes, or haptoglobins (Corning Special Agarose Plates) and most of the separations using agarose date from this time. Examples include serum proteins (Jeppsson, Laurell and Franzén, 1979) and isoenzymes such as lactate dehydrogenase (Yasmineh et al., 1978; Hollaar and van der Laarse, 1979; Papadopoulos, 1981), creatine kinase (Levy and Lum, 1975; Hamilton et al., 1979) and alkaline phosphatase (Rogers, 1976). Surprisingly, Knob and Seidl (1980), studying canine serum creatine kinase isoenzymes, appear to have published the only report of the use of agarose for isoenzyme separations in veterinary medicine.

Polyacrylamide gel

Polyacrylamide is an inert, synthetic medium

prepared by polymerising the monomeric form of acrylamide with methylene bisacrylamide, which acts as a cross-linking agent, to form a rigid gel matrix (Harris and Hopkinson, 1976). The polymerisation process can be initiated either photochemically or chemically.

Photochemically-initiated polymerisation depends on the photodecomposition of riboflavin by ultraviolet light in the presence of traces of oxygen (Gordon, 1969). The ribose groups of riboflavin act as reducing agents so that partial conversion of the flavin to the leuco-form occurs. In the presence of traces of oxygen, the leuco-form is reoxidised with the simultaneous production of free radicles. Polymerisation of the acrylamide monomer, which can only occur in the presence of free radicles, is initiated, and continues due to free radicle production by the acrylamide itself.

In chemically-initiated polymerisation, two substances are required - a base (NNN'N' - tetramethylethylenediamine)(TEMED) or β-dimethylaminopropionitrile (DMAPN) and an initiator (ammonium persulphate). The free oxygen radicles which are produced from persulphate by a base-catalysed reaction transform enough of the acrylamide monomer to the free-radicle state to initiate polymerisation. The presence of more than traces of oxygen inhibits polymerisation (Gordon, 1969). TEMED and ammonium persulphate are

known to adversely affect certain protein molecules, but they can be removed by soaking the gel in buffer or by electrophoresis prior to application of the samples. There are usually no charged groups in polyacrylamide gels, giving them the lowest electro-endosmotic properties of all the support media (Gordon, 1969).

Like starch gel, polyacrylamide gel can exert a molecular sieving affect, and simply by varying the concentration of acrylamide and bisacrylamide, a wide range of pore sizes can be obtained. Pore size may be chosen to provide optimum resolution of any protein (Ornstein, 1964). The superior resolution obtainable by polyacrylamide compared with starch gel has been attributed to the large range of pore diameters present in any one polyacrylamide gel. In general, fine resolution is obtained using a pore diameter which is half the average size of the proteins undergoing separation. In an electric field, proteins will 'squeeze' through pores which they would not diffuse through passively.

Highly symmetrical band shapes are obtainable because of the absence of protein-gel interaction such as ion-exchange effects or adsorption onto the gel.

The absence of reactive groups renders polyacrylamide suitable for a wide variety of protein and enzyme staining methods (Gordon, 1969) and the transparency of this medium is ideal for scanning densitometry

of stained bands (Wilkinson, 1970). However, absorption of ultraviolet light by the gels makes the detection of ultraviolet-absorbing substances *in situ* impracticable (Gordon, 1969).

Although the polymerised form of acrylamide is safe to handle, the monomer is a neurotoxin and several cases of poisoning have been reported (Garland and Patterson, 1967).

Polyacrylamide gel was introduced as a support medium by Raymond and Weintraub in 1959. They found the resolution to be superior to that of other media and noted, when separating serum proteins, that several of the γ-globulin zones were separated into subcomponents. Raymond and Weintraub used flat gels supported vertically during electrophoresis. In the same year, Ornstein and Davis carried out polyacrylamide electrophoresis in cylindrical tubes, a process called "disc electrophoresis" to denote the *disc*-shaped protein zones obtained and the *discontinuous* buffer system used. The theory of the technique was described in detail by Ornstein (1964). The discontinuous buffer system (different buffers in the gel and the electrode chambers) consists of a dilute buffer in a thin "spacer" gel situated above, and in contact with, the electrophoresis gel. The sample is applied to the top of the spacer gel. When the protein bands in the dilute buffer enter the electrophoresis gel

containing a more concentrated buffer, they are sharpened because they carry a front of increased potential gradient. Ornstein resolved twenty serum protein bands in contrast to the five bands found by Kunkel and Tiselius (1952) using paper electrophoresis.

A procedure for horizontal (flat bed) electrophoresis of serum proteins was introduced by Wieme (1962), which he considered suitable for demonstrating the heterogeneity of enzymes such as lactate dehydrogenase. Allred and Kentel (1968) introduced an elegant 'micro'-method for horizontal polyacrylamide gel electrophoresis of lactate dehydrogenase isoenzymes. More recently, ultra thin horizontal gels have been used which have the advantage that zymograms can be developed very rapidly, retaining the sharpness of the bands (Görg et al., 1980).

Unlike disc gels, flat bed systems allow direct comparison to be made between the electrophoretic mobilities of the samples (Laan, Diaz and Szakaly, 1979) since they can be run simultaneously in the same gel.

In the medical field, polyacrylamide has been used for separating the isoenzymes of alkaline phosphatase (Smith, Lightstone and Perry, 1968; Kaplan and Rogers, 1969; Warnes, Hine and Kay, 1976; Cooper et al., 1979), lactate dehydrogenase (Goldberg,

1963; Dietz and Lubrano, 1967), ɣ-glutamyl transpeptidase (Azzopardi and Jayle, 1973; Huseby, 1978; Shaw, London and Petersen, 1978) and creatine kinase (Mercer, 1974). Kaplan and Rogers (1969) and Cooper et al. (1979) considered polyacrylamide electrophoresis to be superior to other electrophoretic techniques for alkaline phosphatase isoenzymes.

In the veterinary field, applications include alkaline phosphatase isoenzyme studies in the sheep (Healy, 1974 and 1975a), horse (Froscher and Nagode, 1979), cat (Everett, Duncan and Prasse, 1977) and dog (Nagode, Koestner and Steinmeyer, 1969), lactate dehydrogenase in the sheep (Michálek and Vodrážka, 1977; Briand et al., 1981) and cow (Bogin et al., 1977), and aspartate aminotransferase in the sheep (Campos-Cavieres and Munn, 1973). Polyacrylamide electrophoresis has also been used for the study of genetic variants of isoenzymes of alcohol dehydrogenase (Grell, Jacobson and Murphy, 1965), glucosephosphate isomerase (Tsuboi and Fukunaga, 1971), lactate dehydrogenase (Klose and Spielmann, 1975), and glutamate dehydrogenase (Fawole, 1977).

Gradient gel electrophoresis

In gradient gel electrophoresis in polyacrylamide gels, the protein molecules in the sample pass through a gradient of increasing polyacrylamide concentration producing a progressive sharpening of bands during

electrophoresis which is independent of the width of the starting zone. Rarely used for routine diagnostic purposes, gradient gel electrophoresis has been applied to serum proteins, lipoproteins, catalase, lactate dehydrogenase (Pharmacia, 1979a), esterase (Görg et al., 1980), and alkaline phosphatase (Guilleux et al., 1978).

Detergent gel electrophoresis

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) was introduced by Summers, Maizel and Darnell in 1965. SDS masks the charge of protein molecules due to its own negative charge, allowing protein separation by electrophoresis solely on the basis of molecular size. This method is used mainly in molecular weight determinations by comparing migration distances with known standards.

The incorporation of the detergent Triton X-100 into electrophoretic media was first investigated by Schneidermann in 1965. More recently this detergent, which interacts with the binding between proteins and lipids (Helenius and Simons, 1975) has found a diagnostic application in the improvement of alkaline phosphatase isoenzyme separations on polyacrylamide gels (Hägerstrand and Skude, 1976).

ISOELECTRIC FOCUSING

The history of isoelectric focusing (IEF) dates

back to 1912 when Ikeda and Suzuki separated amino acids from plant proteins in a three-chambered electrolysis cell. The amino acids arranged themselves according to their isoelectric point to form a pH gradient between the electrodes. Williams and Waterman (1929) extended this idea by using a fourteen-compartment cell to separate mixtures of substances in solution. They found that the pH of the portion of the solution containing a substance in maximum concentration approximated to the isoelectric point of that substance.

In 1955, Kolin, using a U-tube apparatus, separated haemoglobin into two bands by placing the sample at the interface between an acidic and a basic buffer. The mixing of the buffers at the interface formed a pH gradient which allowed separation of the two components of haemoglobin according to their isoelectric point. Tuttle (1956) modified Kolin's method to enable several samples to be run in parallel, in vertical glass tubes. He changed the "slope" of the pH gradient by altering the size of the sample at the interface between the two buffers since the "slope" depended on the distance between the buffers.

Svensson (1961) laid the foundations of modern isoelectric focusing systems by introducing the concept of "natural" and "artificial" pH gradients.

Ampholytes (electrolytes with both acidic and basic properties) in an electrolysis cell such as that described by Williams and Waterman (1929) will aquire a positive charge near the anode and a negative charge near the cathode. Thus they will be repelled by both electrodes until, somewhere between the electrodes, they will lose their charges and will remain stationary at their isoelectric point. If the ampholyte diffuses towards one electrode, it will immediately acquire a charge and will be repelled back to its isoelectric point. A mixture of ampholytes in such a system will be separated according to their isoelectric point to form a pH gradient between the electrodes. Since the gradient has been created by the current itself, Svensson applied the term "natural" pH gradient.

"Artificial" pH gradients, such as those created by Kolin (1955) and Tuttle (1956) in the mixing zone between two buffers are unstable because they are subject to changes due to elective migration of the buffer ions.

Svensson (1961) proposed that if an artificial pH gradient could be made up to imitate a natural one, a highly stable gradient would be formed. He showed that the electrokinetic properties of complex ampholytes such as proteins made them highly suitable for isoelectric fractionation whereas simple ampholytes

could be separated according to their isoelectric points in order to form stable pH gradients in which proteins could be separated by the same principle. He called the simple ampholytes "carrier ampholytes". By chosing suitable carrier ampholytes, Svensson considered that it should be possible to obtain pH gradients of any length, but at that time, suitable simple ampholytes were not available.

By 1966, Vesterberg and Svensson had developed a system of carrier ampholytes which formed a pH gradient capable of separating proteins with a difference in isoelectric point of 0.06 pH units, in a sucrose density gradient column. They showed that the resolving power improves with the shallowness of the pH gradient and with the field strength. This method was used by Carlström and Vesterberg (1967) to demonstrate the heterogeneity of lactoperoxidase and by Weller, Heaney and Sjogren (1968) for lactate dehydrogenase and isocitrate dehydrogenase.

Fawcett (1968) and Dale and Latner (1968) working independently, adapted the technique of Vesterberg and Svensson (1966) to form pH gradients in polyacrylamide disc gels, while Awdeh, Williamson and Askonas (1968) used thin layer polyacrylamide gels and Riley and Coleman (1968) employed agarose as the support medium.

Factors affecting protein separations by isoelectric focusing

Properties of carrier ampholytes

In 1966, Vesterberg and Svensson synthesised a mixture of polyaminopolycarboxylic acids for use as carrier ampholytes which led to the product called Ampholine(R) (LKB Instruments Ltd., South Croydon, Surrey), first marketed in 1967. Pharmalyte(R) (Pharmacia Ltd., London) which became available in the late 1970's is synthesised by the condensation of glycine, glycylglycine, amines with selected pK's (the pK is equivalent to $\log_{10}$ of the reciprocal of the dissociation constant of an electrolyte) and epichlorohidrin (Williams and Söderberg, 1979). Despite differences in chemical structure, Ampholine and Pharmalyte have similar properties and both are available in a variety of pH ranges.

(i) Conductivity

A carrier ampholyte should have a good conductivity at its isoelectric point since regions of low conductivity in the pH gradient result in local overheating and a reduction in the field strength, so reducing the resolution in other areas of the gradient (Righetti and Drysdale, 1976).

Since, by definition, ampholytes have both acidic and basic groups, they have two pK values, pK_1 and pK_2. The relationship between the isoelectric point

(pI) and the pK's is

$$pI = \frac{pK_1 + pK_2}{2}, \quad \text{where } pK_1 \text{ is smaller than } pK_2$$

Svensson (1961) devised an equation to calculate the degree of ionisation of the ampholyte, α:

$$\alpha = \frac{2}{2 + 10^{(pI - pK_1)}}$$

Since the conductivity of an ampholyte at its isoelectric point is directly related to degree of ionisation, the conductivity will be at a maximum when $pI - pK_1$ is at a minimum. Hence, good carrier ampholytes should have pK values as close as possible to their isoelectric point.

(ii) Buffering capacity

Svensson (1962a) showed that the buffering capacity of an ampholyte, $\frac{dQ}{d(pH)}$ is also at a maximum when $pI - pK_1$ is at a minimum, so ampholytes with good conductivity will also have a good buffering capacity.

The buffering capacity must be sufficient to overcome any effect of focused proteins on the pH gradient, and must be even across the pH gradient because of its effect on gradient stability (Williams and Söderberg, 1979).

(iii) Freedom from biological effects

Clearly, ampholytes should not bind to proteins as this could result in artefactual heterogeneity. Dean and Messer (1975) using ^{14}C labelled ampholytes failed to demonstrate ampholyte-protein binding but

Hare, Stimpson and Cann (1978) considered that it contributed to the apparent heterogeneity of acidic wool protein, microbial proteases, bovine serum albumin and tRNA. In the vast majority of cases, however, there is no evidence of irreversible binding (LKB, 1979).

Resolving power of isoelectric focusing

The minimum difference in isoelectric point necessary to separate two protein bands, ΔpI, may be calculated from the equation derived by Svensson (Rilbe, 1976):

$$\Delta(pI) = 3\left[\frac{D(dpH/dx)}{E(-du/dpH)}\right]^{\frac{1}{2}}$$

where D = diffusion coefficient of the protein

E = field strength (V/cm)

dpH/dx = pH gradient

du/dpH = mobility slope of the protein

The diffusion coefficient and the mobility are fixed values for any one protein therefore the only means of increasing the resolution i.e. decreasing ΔpI, is by increasing the field strength or using a narrow pH range (low dpH/dx).

Two factors tend to counteract the zone-sharpening effect of increasing the field strength. Firstly, if the protein concentration is increased by sharpening the protein band to the extent that the intensity of staining is no longer proportional to the protein concentration, then the zone sharpening will not show

up as increased stain intensity, even if it is actually present. Secondly, zone sharpening may be counteracted by the buffering capacity of the sample protein. Where the protein concentration is high, the buffering effect of the ampholytes may be insufficient to prevent the protein forming a small pH plateau at its isoelectric point, allowing the protein to diffuse a short distance without acquiring a net charge. Both of these effects can be overcome by diluting the sample (Låås, Olsson and Söderberg, 1980).

The highest resolution reported is 0.0025 pH unit, (Allen, Harley and Talamo, 1974) but in most isoelectric focusing systems a resolution of 0.01 pH unit is achieved.

pH gradient instability

The instigators of isoelectric focusing using pH gradients formed at the interface between two buffers (Kolin, 1955; Tuttle, 1956) recognised that the gradient was unstable with time. In 1961, when Svensson, using sucrose density gradients, introduced the concept of focusing in natural pH gradients this problem appeared to be solved, and he regarded them as being "characterised by a complete stability". This stability has not been achieved in practice.

pH gradient instability was first recognised in polyacrylamide gels (Findlayson and Chrambach, 1971) although it is now known to exist in the original

sucrose density gradients (Rilbe, 1977). Findlayson and Chrambach proposed the term "plateau phenomenon" to describe the progressive flattening of the pH gradient in the centre of the gel and a steepening of the gradient at both ends of the gel. They showed that the migration of acidic protein bands towards the anode and basic bands towards the cathode occurs concomitantly with the changes in the gradient. Righetti and Drysdale (1971) however, described gradient instability as its "cathodic drift".

Many explanations have been put forward to account for gradient instability (Chrambach et al., 1973; review by Haglund, 1975; Rilbe 1977), but it appears to be of multifactorial origin. Five explanations have been proposed (after Righetti and Drysdale, 1976):

(1) electrophoretic migration of ampholytes from their isoelectric point

(2) electroendosmosis

(3) formation of a zone of pure water in the neutral region of the pH gradient due to neutralisation of hydroxyl and hydronium ions or to accumulation of water at its isoelectric point, with consequent backflow towards the periphery of the gel

(4) deficiency of neutral ampholytes in the ampholyte mixture or progressive destruction of neutral ampholytes

(5) progressive gain or loss of charged ligands by ampholytes.

Whatever the reasons for pH gradient instability, it can be reduced by increasing the carrier ampholyte concentration (Rilbe, 1977) and limiting the field strength (Chrambach et al., 1973). While the latter limits the resolution obtainable, in practice pH gradient instability does not normally adversely affect the reproducibility of isoelectric focusing, particularly in thin layer gels where the focusing time is short (Haglund, 1975).

Support media for analytical isoelectric focusing

Sucrose density gradients

Differences in the density of the solution in which ampholytes are dissolved stabilizes protein zones and minimises convection (Latner, 1975). Sucrose density gradients in U-tubes were the original systems used for isoelectric focusing (Kolin, 1955; Tuttle, 1956) and several elegant techniques have been described for their use for analytical purposes (Weller et al., 1968). However, since the introduction of density gradient isoelectric focusing in vertical glass tubes in 1962 (Svensson, 1962b), the use of U-tubes has been almost abandoned, vertical columns having the advantages of relative ease of recovery of focused zones and measurement of the pH gradient

but problems associated with convective mixing, precipitation of proteins at their isoelectric point, and loss of resolution by diffusion, mixing on elution and the collection of fractions with a greater volume than that of the focused zones, together with the inconvenience of assaying enzyme activity in a large number of eluted fractions has limited the general applicability of the technique (Righetti and Drysdale, 1976) and it is now considered to be inferior to focusing in solid support media.

Polyacrylamide gel

In 1968, Fawcett, and Dale and Latner introduced cylindrical (rod) polyacrylamide gels as a support medium for isoelectric focusing. In the same year, focusing in thin layers (flat beds) of polyacrylamide was first described (Awdeh, Williamson and Askonas, 1968; Leaback and Rutter, 1968). Both papers described the advantages of polyacrylamide over liquid media, i.e. superior resolution, resistance to convective mixing, resistance to diffusion of zones, lack of precipitation of proteins at their isoelectric point, ease of sample application, and suitability for quantitation of stained protein bands by densitometric scanning. In addition, the measurement of the pH gradient is more accurate in solid media since measurements can be made *in situ*.

Several authors have discussed the advantages of flat bed over rod gels and these have been reviewed by Vesterberg (1972):

(1) The time consuming handling of many gel rods is avoided.

(2) The gels are less likely to be scratched or broken.

(3) The flat bed gel allows variation of length and thickness of the gel, as well as the number of samples which may be run simultaneously.

(4) All samples are focused under identical conditions and, as they can be run side by side, comparison is simple.

(5) The flat bed gel is easier to handle for pH measurement, staining, destaining and sectioning.

(6) The result with the flat bed gel may be conveniently recorded by photography and densitometric evaluation.

The fact that field strength improves the resolution in isoelectric focusing has already been discussed. Since the field strength which can be allowed is influenced by the efficiency of heat transfer from a flat bed gel to the cooling plate beneath, which in turn is dependant on the thickness of the gel (Vesterberg, 1975), it may be concluded that flat bed gels will allow a higher field strength to be used than rod gels where the cooling is less

efficient. Thus, greater resolution can be expected with flat bed gels (Bours,1971). In addition, higher field strengths allow shorter focusing time which may be important for labile enzymes.

Possibly the only advantage of gel rods over flat bed gels is that oxygen-sensitive enzymes may be run under anaerobic conditions in rods (Righetti and Drysdale, 1976).

Recently, focusing in ultrathin layers of polyacrylamide gel has been described, and is reported to have advantages over conventional 1-2mm flat bed gels. Görg, Postel and Westermeier (1978) described a method for focusing in 0.12 to 0.36mm gels cast on a cellophane backing. They found that 0.24 and 0.36 mm gels required shorter staining and destaining times with less diffusion of focused zones during staining, and had improved cooling efficiency allowing the use of higher field strengths with a subsequent improvement in resolution. However, in 0.12mm gels, stained bands were distorted, possibly due to electro-endosmosis or interaction of proteins with the cellophane backing.

The properties of polyacrylamide are not utilized in exactly the same way in isoelectric focusing as in electrophoresis. In both techniques the low electroendosmotic properties and chemical inertia are desirable but only in electrophoresis are the molecular sieving properties advantageous. In isoelectric

focusing, the pore size of the gel should be larger than the diameter of the largest protein of interest to avoid retardation of molecules resulting in failure to reach their isoelectric point during the life-span of the pH gradient (Baumann and Chrambach, 1976).

Since the early days of isoelectric focusing, it has been recognised that many more zones of enzyme activity are resolved than in zone electrophoresis (Dale and Latner, 1968). There are numerous reports of attempts to correlate electrophoretic isoenzyme patterns with isoelectric focusing patterns, the vast majority of workers using polyacrylamide gel for both techniques. Chamoles and Karcher (1970a and b) compared tissue lactate dehydrogenase patterns using electrophoresis and isoelectric focusing and described the high degree of heterogeneity produced by the latter. They remarked that each tissue gave a more tissue-specific pattern after focusing than after electrophoresis but the diagnostic implications of this observation have never been fully investigated.

Band patterns on electrophoresis and isoelectric focusing have been compared for aldolase (Yeltman and Harris, 1977), alkaline phosphatase (Gerbitz, Klob and Wieland, 1977), creatine kinase (Cattan, Jamieson and Milner-White, 1978; Chapelle and

Heusghem 1980; Yasmineh, Yamada and Cohn, 1981), glucosephosphate isomerase (Payne, Porter and Gracy, 1972; Mo, Young and Gracy, 1975), aspartate aminotransferase (Campos-Cavieres and Munn, 1973), γ-glutamyl transpeptidase (Tate and Meister, 1976) and enolase (Asaga and Konno, 1975). The findings of some of these authors and their explanations for the existence of sub-banding of isoenzymes have been discussed in Chapter 2.

Agarose

The properties of agarose made it an obvious choice as a support medium for isoelectric focusing since, at a 1% concentration, it combines mechanical stability with almost unhindered migration of macromolecules. However, early attempts were unsuccessful (Riley and Coleman, 1968) due to the presence of sulphate and carboxyl groups in the agarose available at that time which produced severe electroendosmosis, with consequent disruption of the pH gradient. Modification to reduce electroendosmosis (Laas, 1972; Johansson and Hjertén, 1974) resulted in a suitable medium, but it was not used extensively until Saravis and Zamcheck (1979) recommended a commercially available agarose, Isogel (R) (Marine Colloids Division of FMC Corporation, Rockland, ME, U.S.A.). Using a flat bed technique, Saravis and

Zamcheck focused human serum and tissue proteins. In the same year, Rosén, Ek and Åman reported the use of agarose isoelectric focusing for the study of human immunoglobulins. Both papers described the advantages over polyacrylamide gel:

(1) Agarose gels are rapidly formed by a simple gelling process.

(2) Molecular sieving is avoided and macromolecules with a molecular weight of over 2 million daltons are not restricted.

(3) Staining and drying of gels is rapid.

(4) Agarose is non-toxic.

(5) Agarose is suitable for preparative purposes i.e. for recovery of focused proteins by elution from the gel.

Rosén et al. (1979) found the resolving power of agarose comparable to that obtained in thin layer polyacrylamide gel.

There appear to be only two reports on the use of agarose isoelectric focusing for enzyme separations. Cantarow et al. (1982) described a method for the separation of several proteins including alkaline phosphatase, peroxidase and alcohol dehydrogenase, but this was for preparative rather than analytical purposes while Ebert (1982) used the method to separate multiple forms of phosphoglucomutase in trypanosomes.

Cellulose acetate

A method for isoelectric focusing on cellulose acetate strips has been developed (Ambler, 1978a). Treatment with boron trifluoride in methanol reduced the electroendosmotic properties to produce a medium suitable for low voltage focusing. Ambler (1978b) described the separation of human serum alkaline phosphatase and lactate dehydrogenase isoenzymes and serum proteins on cellulose acetate but he found it necessary to concentrate the serum 4-fold and to fix the enzymes on the strip by "salting out" using ammonium sulphate. In the case of lactate dehydrogenase, which is inactive when salted out, the ammonium sulphate had to be removed by washing the strip in buffer before staining.

Righetti and Gianazza (1980), reviewing new developments in isoelectric focusing, considered that the electroendosmotic properties of cellulose acetate are still too high and that it is not a suitable medium for this purpose at the present time. Consequently, it has found few applications.

The measurement of pH gradients in isoelectric focusing gels.

Three methods have been described for the measurement of pH gradients in flat bed gels - elution of ampholytes from gel slices, calibrated pH markers and surface electrodes.

Elution of ampholytes from gels with distilled water,

followed by measurement of the pH of the supernatant is subject to interference from atmospheric carbon dioxide. In addition, pH variations may occur if the temperature at which focusing is carried out and the temperature at which the pH gradient is measured, differ. Dilution with water during elution from the gel is also known to result in inaccuracies (Beeley, Stevenson and Beeley, 1975).

Calibrated isoelectric point markers covering a wide pH range have only recently become commercially available (BDH Chemicals, 1981). The markers consist of mixtures of eight coloured proteins of known isoelectric point. They provide a rapid, accurate and reproducible means of pH gradient determination and are independant of temperature since the markers and samples are focused in the same gel.

Drysdale (Righetti and Drysdale, 1976) developed a flat membrane microelectrode for use on flat bed gels. This y-shaped electrode contains a reference electrode in one arm and the measuring membrane in the other arm, the two arms being 3mm apart. The measuring membrane is 2.5mm in diameter. The electrode is attached to a sliding arm adjacent to a calibrated scale or alternatively, a sheet of graph paper is placed under the gel to form a template. The pH readings are taken at 0.5 or 1.0cm intervals across the gel.

Isoelectric point values obtained by isoelectric

focusing are temperature dependant. This variation may be up to 0.67 pH units between 4 and 25°C (Fredriksson, 1977) and is especially marked for proteins with basic isoelectric points. pH measurements should, therefore be made at a constant temperature, preferably at the focusing temperature (Righetti and Drysdale, 1976).

TWO DIMENSIONAL TECHNIQUES

By combining isoelectric focusing with other procedures such as electrophoresis, it is usually possible to obtain more information than is available from either procedure alone (Righetti and Drysdale, 1976). In general, focusing is used as the first dimension - the gel rod or strip of flat bed gel containing the focused protein zones is embedded on top of the second dimension gel and the focused zones are separated by electrophoresis in the second dimension gel. For isoenzyme studies, the second gel may be a conventional gel for zone electrophoresis or a gradient gel.

Two dimensional techniques are of value in correlating electrophoretic isoenzyme patterns with those observed after focusing (Hayes and Wellner, 1969). More recently, Klose and Spielmann (1975) investigated the microheterogeneity of mouse tissue lactate dehydrogenase.

CONCLUSIONS

The review of the literature on methods of separating isoenzymes indicated that some techniques were more suitable than others for quantitative determinations. Techniques considered suitable for further study are listed in Table 3.1.

TABLE 3.1.

METHODS SELECTED FOR FURTHER STUDY

Method	Reason	Reference
Thin layer zone electrophoresis	Allows quantitation of all isoenzymes in a mixture simultaneously.	
	Allows several samples to be run simultaneously.	Laan, Diaz and Szakaly (1979). Fritz et al. (1970).
	Suitable for rapid quantitation by visualising isoenzymes in situ.	Rosalki (1974a)
(a) in agarose	Good resolution.	
	Optically clear gel suitable for densitometric or fluorometric scanning of stained isoenzyme bands.	Pharmacia (1978) Papadopoulos (1981)
	Commercially prepared plates available.	Corning Medical
(b) in polyacrylamide	Superior resolution to other media.	Dietz and Lubrano (1967)
	"	Gordon (1969)
	Optically clear gel suitable for densitometric scanning.	Dietz and Lubrano (1967)
	"	Wilkinson (1970)
Thin layer IEF		
(a) in agarose (b) in polyacrylamide	Reported to give a similar degree of resolution. Both media superior to cellulose acetate.	Rosén et al. (1979) Righetti and Gianazza (1980)

CHAPTER 4

DEVELOPMENT OF TECHNIQUES

PART I

STAINING AND QUANTIFYING ISOENZYMES AFTER ISOELECTRIC FOCUSING AND ELECTROPHORESIS.

INTRODUCTION

Enzyme activity in gels may be detected by elution of the isoenzymes from segments of the gel followed by assay of the fractions by conventional means for measuring total enzyme activity, or by staining of isoenzyme bands *in situ* followed by quantitative densitometric or fluorometric scanning. Various authors including Wilkinson (1970) have discussed the advantages and disadvantages of elution, while Hunter and Burstone (1958) and Wieme and Van Maercke (1961) describe *in situ* enzyme detection.

The combination of dyes to the products of an enzyme-catalysed reaction can be achieved in a variety of ways. Decker and Rau (1963) describe the use of Fast Violet B salt to combine with oxaloacetate in the detection of aspartate aminotransferase. Tetrazolium salts were first introduced in 1949 by Kun and Abood. Methyl thiazolyl blue and Nitro blue tetrazolium (NBT) form coloured formazans by indirect combination with the $NADH_2$ or $NADPH_2$ formed during reactions involving NAD(P) - dependant enzymes. The reaction occurs indirectly

via hydrogen carriers such as Meldolablue. If the enzyme of interest is not NAD(P) - dependant, the reaction must be linked to a reaction involving an enzyme which is NAD(P) - dependant. Tetrazolium staining procedures are used to detect the isoenzymes of lactate dehydrogenase (Markert and Møller, 1959), aldolase and glucosephosphate isomerase (Harris and Hopkinson, 1976).

Tetrazolium staining has the disadvantage that non-specific bands of coloured formazan may be produced even in the absence of substrate. This is known as the "nothing dehydrogenase" effect (Pearse, 1961), and only tetrazolium and the hydrogen carrier are required to produce it (Somer, 1975). Several enzymes have been incriminated as being responsible for this effect. Whatever the reason for its existence, the possibility of interference by "nothing dehydrogenase" with zymograms produced by tetrazolium staining should be investigated by comparing stained gels with "blank" preparations from which the substrate has been omitted. Somer (1975) stated that such interference can be avoided by using fluorescent methods of visualising isoenzymes.

Fluorescent methods are reported to be more sensitive than tetrazolium salts (Somer and Konttinen, 1972; Mickle, Washington and Porter, 1978) and are free from the "nothing dehydrogenase" effect.

Reaction mixtures are applied to electrophoretic

media in a variety of ways, either as an aqueous solution or in a solid medium which is applied to the gel surface (Markert and Møller, 1959; Wieme and Van Maercke, 1961; Decker and Rau, 1963; Dorner, Hoffmann and Long, 1974).

Densitometric and fluorometric scanning are used to measure the intensity of the end products of tetrazolium and fluorescent staining respectively, and some densitometers are capable of both types of measurement (Beckman, 1975). When reading results, it is essential that linearity between peak area on the densitometer tracing and increasing concentration of end product be demonstrable (Rosalki, 1974b).

MATERIALS, METHODS AND RESULTS

Lactate dehydrogenase

(a) Fluorescent method

Both electrophoresis and isoelectric focusing gels were stained according to the method of Corning Medical, Palo Alto, California, U.S.A., described by Galen (1974). A stock solution was made up containing 1.8M DL-lactic acid (sodium salt), 0.67M 2-amino-2-methyl-1, 3-propandiol (AMP) and 20% sucrose (all from Sigma Chemical Company, Poole, Dorset). The reaction mixture consisted of the following:

lactate/AMP/sucrose stock solution - 40ml

NAD (Sigma) - 40mg

'Ionagar' No.2 (Oxoid Division of Oxo Ltd., London) - 0.4g

The mixture was applied as an agar overlay on gels cast on glass plates for initial, qualitative investigations. Gels were incubated in the dark at 37°C for 20 minutes in a hot air oven (Laboratory Thermal Equipment Ltd., Greenfield, Oldham). For qualitative assessments, gels were examined under a Model UVL-21 Blak-Ray Lamp (Ultra-Violet Products, Inc., San Gabriel, California, U.S.A.) with a wavelength of 366nm. The lactate dehydrogenase patterns obtained by isoelectric focusing and electrophoresis of ovine tissue homogenates are shown in Figs. 4.1 and 4.2. All figures for Chapter 4 are in Appendix 1.

The specificity of staining was investigated by running three samples in duplicate on an electrophoresis plate, staining one set of samples in the absence and the other in the presence of lactate (Fig. 4.3). Staining was shown to be specific for lactate dehydrogenase and the "nothing dehydrogenase" effect was not observed. This was repeated on three different occasions and the same results were obtained.

Gels were scanned for isoenzyme quantitation in a Beckman R-112 scanning densitometer with a fluorometric attachment (Beckman Instruments Inc., Clinical Instruments Division, Fullerton, California, U.S.A.).

Gels were cast on GelBond film (FMC Corporation, Marine Colloids Division, Rockland, Maine, U.S.A.),

a 0.2mm thick, transparent film with a hydrophobic and a hydrophilic surface. Gels adhere to the hydrophilic surface. This film was used to allow gels to be cut to a suitable size to fit into the densitometer. However, GelBond film was found to fluoresce under ultraviolet light, masking the fluorescence of the isoenzyme bands. By cutting the gels into sections of 9cm x 9cm and placing them on 1.5mm thick glass plates, 10cm x 10cm in size, fluorometric scanning was possible. This was a very difficult procedure because of the fragility of the polyacrylamide gels and many gels were lost during the transfer. Although glass was non-fluorescent, it contained impurities which were detected by the densitometer as fluorescence and produced peaks on the pen tracing, making the results difficult to interpret and quantitatively inaccurate. The reaction mixture was then applied as a filter paper overlay using Whatman No. 1 filter paper (Whatman LabSales Ltd., Maidstone, Kent), in an attempt to improve the results obtained by fluorometric scanning. After incubation, the filter paper was removed, dried with a 2 kilowatt convection heater, and cut into strips for scanning. Although fluorescence was much more intense on the dried filter paper strips than on gels, uneven "background" fluorescence was detected and the non-fluorescent areas between fluorescent bands were

frequently recorded on the chart paper as being below the baseline, making quantitation impossible.

In addition, the fine resolution obtained by isoelectric focusing was lost when filter paper overlays were used, the enzyme bands being fewer in number and more diffuse than in the original gel. The use of finer grades of filter paper (Whatman No. 44 and Whatman No. 50) or cellulose acetate membranes (Cellogel(R), Whatman LabSales Ltd.; Sepraphore(R), Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.) for applying the reaction mixture did not improve the resolution. Consequently, attention was turned to the tetrazolium staining method as a means of visualising lactate dehydrogenase isoenzymes.

(b) Tetrazolium method

Initially, both isoelectric focusing and electrophoresis gels were stained by the method of Kubicz and Wolanska (1977), modified to include Meldola-blue instead of phenazine methosulphate and methyl thiazolyl blue (MTT) (Sigma) instead of p-iodo-nitrotetrazolium violet. Gels were placed in an aqueous solution containing the reaction mixture and incubated in a hot air oven at 37°C for times varying from 1 to 16 hours. Staining appeared to be pale despite prolonged incubation. The reaction mixture was modified to increase the concentration of lactate, NAD and MTT to give the following composition:

0.1M phosphate buffer, pH 7.5	- 30ml
1M sodium lactate	- 8ml
NAD	- 80mg
MTT	- 24mg
Meldolablue (6mg/ml)	- 200µl

The staining intensity was markedly increased when this reaction mixture was used. Resolution of bands was improved by applying the reaction mixture as a molten agar overlay which was allowed to harden on top of the isoelectric focusing or electrophoresis gel. This was prepared by dissolving 0.4g 'Ion-agar' No. 2 in 30ml of phosphate buffer and adding the solution containing the sodium lactate, NAD, MTT and Meldolablue immediately before pouring onto the surface of the polyacrylamide, the gel being surrounded by a template of the same dimensions as the gel. The improved resolution obtained by the use of agar overlays is demonstrated in Fig. 4.4.

The specificity of the staining method for lactate dehydrogenase was investigated by running three samples in duplicate and incubating one set of samples in the presence and the other in the absence of lactate. Fig. 4.5 demonstrates that pale staining was visible in the absence of substrate, the pattern appearing to correspond exactly to the lactate dehydrogenase pattern in the samples. Since the "nothing dehydrogenase" effect appeared to be due to

lactate dehydrogenase activity in the samples, it was considered to be unimportant when staining for this enzyme.

To assess the suitability of the tetrazolium staining technique for densitometric scanning, both electrophoresis and isoelectric focusing gels were cast on GelBond film. After staining, gels were cut into strips and scanned at 550nm in the Beckman R-112 scanning densitometer. The uneven background staining produced by the fluorometric method was not observed with tetrazolium staining, peaks on the pen tracing consisting of a series of smooth curves corresponding to the bands of enzyme activity and it was possible to quantify the isoenzyme bands obtained by electrophoresis. However, scanning of isoelectric focusing gels using this instrument produced two problems. Firstly, minor bands which were visible to the naked eye were not always detected and secondly, the closely stacked bands, which appear to be well separated visually, revealed overlapping or fusion on the tracing, making quantitation of individual bands impossible. Thus, the tetrazolium method was more suitable than the fluorescent method for qualitative purposes in both electrophoresis and isoelectric focusing gels and for quantitative purposes in electrophoresis gels and was used for subsequent work on lactate dehydrogenase. However

neither fluorometric nor densitometric scanning of isoelectric focusing gels using the Beckman R-112 densitometer produced scans suitable for quantitation and the fine resolution obtainable by the isoelectric focusing technique was not recorded by the instrument.

In the absence of a suitable densitometer, quantitative studies of multiple forms of lactate dehydrogenase isoenzymes by isoelectric focusing were not pursued further.

For reasons which have already been discussed, it was necessary to determine the level of lactate dehydrogenase activity at which the peak area on the densitometric scan, after tetrazolium staining, was no longer directly proportional to the enzyme activity. Five serial dilutions of an ovine serum sample with a total lactate dehydrogenase level of 649.0 IU/l were made in 0.25M sucrose. Samples were applied as a narrow band to a polyacrylamide gel electrophoresis plate by means of a 1.5µl Cellogel applicator (Whatman LabSales Ltd.) since this was the quantity used for electrophoresis. The series of five dilutions were applied in eight separate columns on one electrophoresis plate.

After allowing the samples to soak into the gel for 30 minutes, the gel was stained by the tetrazolium method, each of the eight columns of five

dilutions was scanned and the area under each peak calculated as a percentage of the total of the peak areas for that column. The results are shown in Fig. 4.6. Within the limits of the standard deviations, peak area is directly proportional to enzyme activity to a level of approximately 400 IU/l. Consequently, on the assumption that all the enzyme activity of a sample might be concentrated in one band, all samples with a lactate dehydrogenase level of more than 400 IU/l were diluted before electrophoresis.

The results obtained by densitometric scanning of lactate dehydrogenase zymograms after isoelectric focusing appear to be similar to those of Chamoles and Karcher (1970b), with marked overlapping of peaks on the densitometer tracing. The authors did not attempt to quantify the multiple bands.

Zeineh (1977) has recommended the use of a soft laser scanning densitometer with a 50μ wide laser beam for scanning gels after focusing whereas the Beckman R-112 densitometer has a minimum light source width of 300μ. He considered that the resolution obtainable from conventional scanning densitometers was not compatible with that of isoelectric focusing.

Aldolase

(a) Tetrazolium method

Electrophoresis and isoelectric focusing gels were stained by a modification of the method of

Harris and Hopkinson (1976) as an agar overlay:

0.1M Tris HCl buffer, pH 8.0	- 30ml
fructose-1,6-diphosphate (trisodium salt) (Sigma)	- 200mg
NAD	- 30mg
$NaHAsO_4$ (BDH Chemicals Ltd., Poole, Dorset)	- 36mg
glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle) (Boehringer Mannheim GmbH)	- 24 units
MTT	- 9mg
Meldolablue (6mg/ml)	- 200µl
'Ionagar' No. 2	- 0.3g

The 'Ionagar' was dissolved in 20ml of Tris HCl buffer and the other constituents added to the remaining 10ml buffer. The agar solution was allowed to cool to 45°C before addition of the reaction mixture to avoid denaturation of the glyceraldehyde-3-phosphate dehydrogenase. Gels undergoing staining were incubated in the dark at 37°C for 1½ hours.

The specificity of staining was investigated by staining one set of duplicate samples in the presence and the other in the absence of fructose-1,6-diphosphate. Faint bands resembling the expected lactate dehydrogenase pattern for each sample were observed, confirming the views of Silverstein and Geller (1974) (Fig. 4.7.) A diffusely stained area anodal to the origin was not observed in the absence of substrate and presumably represented aldolase activity. Attempts were made to eliminate non-specific staining using the lactate

dehydrogenase inhibitors silver nitrate ($Ag\ NO_3$) and oxalate (oxalic acid, sodium salt, Sigma) at concentrations used by Silverstein and Geller. Six samples of an ovine heart muscle homogenate were subjected to electrophoresis. The plate was then cut into five strips and stained as follows:

1. Aldolase reaction mixture including substrate.
2. " " " " " + 300µM $AgNO_3$
3. " " " " " + 15mM oxalate
4. " " " without "
5. " " " " " + 300µM $AgNO_3$
6. " " " " " + 15mM oxalate

This was repeated on three occasions and the results in each case were as follows:

1. Staining just anodal to origin and approximately 4cm anodal to origin.
2. No staining.
3. No staining.
4. Faint staining approximately 4cm anodal to origin.
5. No staining.
6. No staining.

The faint staining observed in the absence of substrate (sample 4) was in the expected position of LDH_1, the predominant lactate dehydrogenase isoenzyme in heart muscle, while the staining near the origin (sample 1) was considered to be due to aldolase activity since it was only observed in the presence of fructose-1,6-

diphosphate. Neither aldolase nor non-specific enzyme activity was visible in the presence of the lactate dehydrogenase inhibitors $AgNO_3$ and oxalate. The inhibition of aldolase activity clearly rendered lactate dehydrogenase inhibition an unsuitable method of eliminating non-specific staining.

(b) Fluorescent method

The reaction mixture was the same as for the tetrazolium method, but with the omission of Meldolablue and MTT, and was applied as an agar overlay during the development of the technique. For fluorometric scanning 'Ionagar' was omitted and the reaction mixture applied as a filter paper overlay, as described for lactate dehydrogenase, followed by incubation in a dark, humid chamber at 37°C for 2 hours.

The fluorescent method was shown to be specific for aldolase by staining duplicate samples in the presence and absence of fructose-1,6-diphosphate. This was repeated three times, and in each case, no non-specific fluorescence was visible in the absence of substrate (Fig. 4.8). The fluorescent method was therefore used for all subsequent work on aldolase.

To determine the point at which peak area on the fluorometric scan was no longer proportional to aldolase activity with the ultraviolet method, five serial dilutions of an ovine serum sample with

a total aldolase activity of 61.9 IU/l were applied to an electrophoresis plate as described for lactate dehydrogenase. The results which are shown in Fig. 4.9 , indicate that peak area is proportional to aldolase activity at least to 60 IU/l. For quantitative studies, all samples with an aldolase activity of more than 60 IU/l were diluted before electrophoresis.

Glucosephosphate isomerase

(a) Fluorescent method

In view of the problems encountered during fluorometric scanning of lactate dehydrogenase, particularly the difficulty in obtaining a baseline, it was decided that if the tetrazolium staining was specific for glucosephosphate isomerase activity, the fluorescent method would not be investigated.

(b) Tetrazolium method

Electrophoresis and isoelectric focusing gels were stained by the method of Harris and Hopkinson (1976), modified to include Meldolablue instead of phenazine methosulphate. The reaction mixture was as follows:

Tris Cl buffer 0.03M pH 8.0	- 40ml
fructose-6-phosphate (barium salt) (Sigma)	- 16mg
glucose-6-phosphate dehydrogenase (Type XI, Sigma)	- 2.4 units
NADP	- 4mg

MTT	- 11mg
Meldolablue (6mg/ml)	- 200µl
'Ionagar' No. 2	- 0.4g

The agar was dissolved in 30ml of Tris Cl buffer and the constituents of the reaction mixture in the remaining 10ml. The agar solution was allowed to cool to 45°C before addition of the reaction mixture to prevent denaturation of the glucose-6-phosphate dehydrogenase. Gels were incubated at 37°C in the dark for 1½ hours and scanned at 550nm.

The specificity of staining was assessed by incubating half of an electrophoresis plate containing duplicate samples in the presence and the other half in the absence of fructose-6-phosphate. This was repeated three times and in each case staining was specific for glucosephosphate isomerase (Fig. 4.10).

Densitometric scanning of isoelectric focusing gels stained for glucosephosphate isomerase produced the same problems as those stained for lactate dehydrogenase, i.e. lack of sensitivity and inability to record closely stacked bands as separate entities. Quantitative studies of the multiple forms of glucosephosphate isomerase after focusing were therefore considered to be impossible using the available equipment. Scanning of electrophoresis gels however, resulted in smooth peaks with little activity recorded between the peaks, producing a result suitable for quantitation.

The level of glucosephosphate isomerase activity at which peak area on the scan was no longer proportional to enzyme activity was determined as described for lactate dehydrogenase.

The results which are shown in Fig.4.11, indicate that peak area and activity are directly proportional over the range of activity tested. For future work, all samples with a glucosephosphate isomerase level of more than 650 IU/l were diluted before electrophoresis.

Aspartate aminotransferase

The staining method was a modification of the method of Decker and Rau (1963):

phosphate buffer 0.2M pH 7.4 - 13.25ml

Fast violet B salt* - 125mg in 4.25ml distilled H_2O

polyvinylpyrrolidone* - 1.875g

pyridoxal-5-phosphate (500µg/ml)* - 0.5ml

bovine albumin fraction V (30mg/ml)+ - 1.0ml

L-aspartic acid* (0.2M adjusted to pH 7.4 with 1M KOH) - 4.25ml

α-oxoglutaric acid* (0.1M adjusted to pH 7.4 with 1M KOH) - 1.75ml

'Ionagar' No. 2 - 0.25g

* Sigma

\+ Miles Laboratories Ltd., Stoke Poges, Slough.

The agar was dissolved in the phosphate buffer, and the other constituents were added immediately

before pouring over the electrophoresis or isoelectric focusing gel. Gels were incubated at 37°C in the dark for 2 hours and scanned at 420nm.

The specificity of staining was determined by incubating samples of ovine liver and heart homogenates in the presence and in the absence of L-aspartic acid in the reaction mixture. Staining was shown to be specific for aspartate aminotransferase (Fig. 4.12).

In the original method described by Decker and Rau, the reaction mixture was applied as a filter paper overlay on starch gel followed by quantitation of isoenzymes by elution from the gel. I found that the stained bands in the polyacrylamide gel after application of the substrate solution by this means were barely detected by the densitometer while scanning of the dried filter paper produced poor resolution and a very uneven densitometric tracing with multiple artefactual peaks. Although aspartate aminotransferase activity was visible when gels were incubated in an aqueous solution of the reaction mixture, the stained areas were on the surface of the gel and disappeared during removal of the gel from the solution. Consequently, it was necessary to apply the reaction mixture as an agar overlay. However, during incubation, a large number of tiny bubbles were produced in the overlay (Fig. 4.31). These produced small peaks when the gel was scanned and the results were impossible to quantify. For this reason,

aspartate aminotransferase was not investigated further.

Creatine kinase

(a) Tetrazolium method

Although a tetrazolium staining method has been developed for creatine kinase isoenzymes (Rosalki, 1965) it was not used in this study because the "nothing dehydrogenase" effect has been reported to interfere with the results (Somer and Konttinen, 1972).

(b) Fluorescent method

Creatine kinase isoenzymes were visualised using the Corning CK Isoenzyme Substrate Kit (Corning Medical, Catalogue No. 470114) according to the manufacturer's instructions. 1ml of 2-(N-Morpholino) ethane sulfonic acid (MES) buffer, pH 6.2 was used to dissolve one vial of the reaction mixture containing the following:

	Final Concentration in Reagent
creatine phosphate	189mM
adenosine diphosphate (ADP)	6mM
Mg^{2+}	33mM
adenosine monophosphate (AMP)	18mM
NADP	6mM
glucose	63mM
glutathione	84mM
hexokinase	9×10^3 IU/l
glucose-6-phosphate dehydrogenase	3×10^3 IU/l

The series of reactions resulting in the production of $NADPH_2$ have been described on pages 83 and 84. AMP is included to inhibit adenylate kinase activity which interferes with creatine kinase determinations (Knob and Seidl, 1980), while glutathione, a sulphydryl compound, acts as an enzyme stabiliser and reactivator.

Electrophoresis was carried out in agarose gels. The reaction mixture was dispensed onto the gel surface along the edge of a 5ml pipette which was placed lengthwise along the cathode edge of the gel, the solution being spread over the plate by moving the pipette across the gel to the anode. The gel was incubated in a moist chamber at 37°C for 20 minutes, then dried in an oven at 60°C for 15-20 minutes to form a thin film on the plastic backing. Drying enhances fluorescence and decreases "background" activity but if gels are over-dried they become opaque.

Fluorescence was shown to be specific for creatine kinase by incubating samples after electrophoresis in the presence and absence of creatine phosphate and glucose. No fluorescence was visible in the absence of the substrates (Fig. 4.13). Fluorometric scanning produced relatively smooth peaks with little or no "background" activity.

The level of creatine kinase above which peak area is no longer proportional to enzyme activity was determined using five serial dilutions of an ovine

serum sample in the manner described for lactate dehydrogenase. 3µl samples of each dilution were applied as this was the quantity of serum used for electrophoresis. The results are shown in Fig. 4.14 . Above approximately 260 IU/l, dilution of the sample is necessary before electrophoresis.

PART 2

DEVELOPMENT OF ISOELECTRIC FOCUSING TECHNIQUES

MATERIALS, METHODS AND RESULTS

Isoelectric focusing in agarose and polyacrylamide gel was carried out on an FBE-3000 Flat Bed Apparatus (Pharmacia Ltd.) consisting of a horizontal aluminium cooling plate covered with teflon cloth, two buffer vessels for use when the apparatus is converted to an electrophoresis system, and a polycarbonate lid fitted with adjustable platinum electrodes (Fig. 4.15). The apparatus was connected to a Chandos PSU 2500V power pack (Chandos Works, Stockport, Cheshire) capable of operation at constant voltage, current or wattage.

Tap water was circulated continuously through a baffle plate beneath the aluminium cooling plate during focusing.

Isoelectric Focusing in Agarose

Preparation of plates

Gels were prepared according to the manufacturers (Pharmacia Ltd.) instructions. The 30ml of gel solution required for one 115x230mm plate contained 0.3g Agarose IEF (Pharmacia Ltd.), 3.6g D-sorbitol (Sigma), 27.0ml of distilled water and 1.9ml Pharmalyte carrier ampholyte (Pharmacia Ltd.). The Pharmalyte was added after dissolution of the agarose by heating

in a conical flask. Pharmalyte pH intervals used were pH 3-10 and pH 8-10.5.

Gels were either cast on 2mm thick glass plates or 0.2mm thick GelBond hydrophilic film, 115x230mm in size. When GelBond was used, it was placed hydrophilic side up on a glass plate with a layer of distilled water between the film and the glass. A casting frame was clamped to the glass plate with bulldog clips. This was placed on a levelled Photax heating plate at 50-60°C. The gel solution was poured onto the glass plate and any air bubbles were burst with a hot needle. After cooling for 10-15 minutes, the frame was removed and the gel allowed to harden fully for at least an hour in a humid chamber at 4°C.

Procedure for focusing

5ml of distilled water was placed on the cooling plate to ensure good contact between the cooling plate and the gel. The electrode strips were soaked in the appropriate electrode solution, blotted for one minute and placed along the long edges of the gel. The anode solution was 0.05M H_2SO_4 for the pH 3-10 gradient and 0.2M L-histidine (Sigma) for the pH 8-10.5 gradient. In both cases the cathode solution was 1M NaOH. Electrode solutions at the anode and cathode prevent oxidation and reduction, respectively, of the sample and carrier ampholytes at the electrodes

(LKB, 1979).

Samples were applied onto the gel surface and the lid was positioned with the electrodes in contact with the electrode strips. For the pH 3-10 gradient, the power pack was set to deliver a maximum of 15W and 1500V with unlimited current for 1½ hours whereas the pH 8-10.5 gradient required 5W, 600V, and unlimited current for 3 hours. The process was followed by observing the focusing of haemoglobin bands applied opposite each other near each electrode wick.

Staining

Prior to staining, gels were dried by placing three layers of Whatman 3MM filter paper on top of the gel, followed by a glass plate and a weight of about 1kg. After 15 minutes, these were removed and the gel dried in an oven at 60°C for 10 minutes. The staining procedures for enzymes have been described. Serum proteins were stained as follows by the method recommended by Bio-Rad Laboratories Ltd., Watford, Hertfordshire. The proteins were fixed by immersion of the gel in a solution containing 4% sulphosalicylic acid and 12.5% trichloroacetic acid for 1 hour then stained in 27% ethanol, 10% acetic acid, 0.04% Coomassie Brilliant Blue R (Sigma) and 0.5% $CuSO_4$ for at least 2 hours. Gels were destained in two or three changes of the first destaining solution consisting of 12% ethanol, 7% acetic acid and 0.5% $CuSO_4$ until the

background was nearly clear, followed by immersion in the second destaining solution comprising 12% ethanol and 7% acetic acid to remove the final traces of stain and $CuSO_4$. The inclusion of $CuSO_4$ in the stain and first destaining solution eliminates any background staining of the ampholytes.

After staining, gels were placed in a hot air oven at 60°C until completely dry.

Preliminary work on serum proteins

Four runs were carried out in a pH 3-10 gradient using serum samples from several domestic species. Samples were applied approximately 0.5cm from the cathode wick (Rosén et al., 1979) directly onto the gel surface either as a 4µl drop or using a 1.5µl Cellqgel sample applicator (Whatman LabSales Ltd.) or as 10-20µl samples soaked into Paratex filter pieces (LKB Instruments Ltd.). The latter were removed 30 minutes after the start of focusing.

Paratex caused sample overloading, longitudinal streaking, band distortion at the point of application and poor resolution of protein zones especially towards the cathode. Samples applied directly to the gel surface showed superior resolution and reduced longitudinal streaking. The best results were obtained when the sample was applied as a 4µl drop (Fig. 4.16).

Lactate dehydrogenase

Agarose isoelectric focusing of lactate dehydrogenase has not been previously reported. To assess the suitability of agarose for focusing of lactate dehydrogenase, four gels containing Pharmalyte pH 3-10 were run. Homogenates of ovine heart, kidney, liver, skeletal muscle, lung, rumen mucosa, small intestine mucosa, large intestine mucosa (1g tissue in 4ml 0.25M sucrose) and serum samples from several species, were each applied as a 4µl drop, 0.5cm from the cathode wick. (For preparation of homogenates see page 180).

In two of the four runs, the samples migrated at an angle and showed longitudinal streaks of enzyme activity between bands, while in all four, bands were poorly resolved (Fig. 4.17). Ovine serum samples showed up to seven bands, while skeletal muscle was the most heterogeneous with fifteen bands. The multiple bands of serum and tissue samples appeared to form five main zones of activity.

Aldolase

Isoelectric focusing of aldolase in agarose gel has not been previously described. Seven runs were carried out in a pH 3-10 gradient under the same conditions and using the same samples as for lactate dehydrogenase. It was necessary to treat the results

obtained with caution since faint non-specific staining in the absence of the substrate fructose-1,6-diphosphate was observed because the tetrazolium staining method was still being used to detect aldolase activity when the isoelectric focusing technique was being developed. However, since the band pattern of the non-specific staining closely resembled the lactate dehydrogenase pattern, it was possible to interpret the results by comparison with the lactate dehydrogenase patterns described above for the same samples. The elimination of non-specific staining was discussed in the section on staining techniques.

In each run, serum samples showed no staining or very faint staining near the cathode which was not due to failure of the aldolase to migrate from the point of application since aldolase activity was observed at the same site when the samples were applied near the anode.

Ovine tissue homogenates migrated diagonally in two of the runs and in four runs staining was diffuse and resolution poor. In three gels however, liver, kidney and heart samples showed sharp zones near the cathode which were different from the non-specific pattern. The number of bands was not consistent - liver aldolase varied between 5 and 9 bands, while kidney showed 6 or 7 bands. Aldolase from the heart formed 4 bands and on one occasion, lung aldolase formed 2 bands. Other tissues appeared to show non-

specific staining.

In an attempt to improve band separation, liver, kidney, heart and skeletal muscle homogenates were re-run in a pH 8 - 10.5 gradient. This was repeated three times using aliquots of the same samples. In two gels staining was diffuse for all four samples (Fig. 4.18). The third gel showed 5 liver bands, 6 kidney bands and 4 skeletal muscle bands whereas the heart sample showed a diffuse area of staining.

The bands were wider apart than on the wide range gradient but resolution was poor with increased staining between bands. However, skeletal muscle aldolase was resolved into distinct zones which were not visible in the pH 3-10 gradient.

Glucosephosphate isomerase

Isoelectric focusing of glucosephosphate isomerase in agarose gel has not been previously described. Two gels containing a gradient of pH 3-10 were run under the same conditions, and using the same samples as described for lactate dehydrogenase. In both cases the bands were poorly resolved (Fig. 4.19). Ovine serum was resolved into six bands and tissue homogenates into 6-9 bands.

Aspartate aminotransferase

In view of the poor results obtained for lactate dehydrogenase, aldolase and glucosephosphate isomerase, isoelectric focusing of aspartate aminotransferase in

agarose gel was not attempted.

Conclusions

Thus, agarose isoelectric focusing was considered to be unsatisfactory for investigating enzyme micro-heterogeneity for the following reasons:

(a) Results for the same sample were inconsistent.

(b) Resolution was poor.

(c) Longitudinal tailing was frequently observed.

(d) Samples tended to run diagonally.

I therefore decided to investigate polyacrylamide gel as a support medium for isoelectric focusing.

Isoelectric focusing in polyacrylamide gel

Preparation of plates

Gels were cast on GelBond film or on glass plates treated with Silane A174 (Pharmacia Ltd.), an organosilane ester which promotes adhesion between the gel and the glass plate. Casting was carried out in a gel moulding stand (Pharmacia Ltd.) basically consisting of a gasket, 1mm thick, between two 115 x 230mm glass plates clamped together with bulldog clips (Fig. 4.15). A piece of hydrophobic polyester film was stuck to one of the glass plates by a few drops of glycerol to ensure that the gel only adhered to the hydrophilic GelBond film or to the Silane-treated glass plate. When GelBond was used, it was stuck

to the other glass plate in a similar manner. The stand held the casting frame vertically during polymerisation. The acrylamide solution was slowly introduced at one corner by lifting the gasket slightly and inserting a 20 gauge, 1½ inch needle attached to a 50ml syringe containing the solution, between the plates.

Initially, gels were prepared according to the manufacturer's instructions. A stock solution was prepared containing 24.25g acrylamide (BDH), 0.75g NN'-Methylenebisacrylamide (BDH), 2.5g 'Amberlite' monobed resin MB-1 (BDH) and 250ml distilled water. The solution was stirred for an hour with a magnetic stirrer, filtered, and kept at 4°C for up to one week. Removal of free acrylic acid by Amberlite is reported to reduce pH gradient drift (Pharmacia, 1979b).

Gel solution for one plate contained 15ml stock solution, 1.9ml Pharmalyte, and 4ml glycerol, made up to 30ml with distilled water. The solution was deaerated in a 100ml conical flask with a vacuum pump (Edwards High Vacuum Ltd., Crawley), at approximately 250 mm Hg to remove excess oxygen which inhibits polymerisation. 200µl ammonium persulphate solution (22.8mg/ml) was added immediately before transferring the solution to the syringe for introduction into the casting frame.

Gels prepared in this manner consistently failed to polymerise despite deaeration for periods of up to 2 hours, and a considerable amount of time was wasted as a result.

Lundstrom and Roderick (1979) described a method of pouring gels for isoelectric focusing which unlike the method published by Pharmacia, was consistent with the concept of chemical polymerisation in the presence of a base (TEMED) and an initiator (ammonium persulphate) (Gordon, 1969). The gel solution contained the following:

distilled water	- 16ml
50% (v/v) glycerol	- 6ml
20% (w/v) acrylamide + 0.8% (w/v) bisacrylamide	- 6ml
TEMED	- 10µl
Pharmalyte	- 1.9ml

This gave a total acrylamide concentration (T) of 4.2% and a percentage of the crosslinker bisacrylamide (C) of 3.8%, the latter being expressed as a percentage of the total acrylamide concentration. The mixture was deaerated for 4 minutes under vacuum, and 100µl 10% (w/v) ammonium persulphate added followed by a further 1 minute deaeration, before introduction into the casting frame. Polymerisation was complete in 20 - 30 minutes.

The gel composition described above was used for preliminary work on serum proteins. Modifications

of this method for individual enzymes are described below.

After polymerisation, the casting frame was dismantled by removing the gasket and prising the glass plates apart with a spatula. The polyester film adherent to the top of the gel was peeled off to leave the gel on the GelBond film or Silane-treated glass plate. Gels were stored in polythene bags at 4°C for a least 12 hours before use.

Procedure for focusing

Gels were placed on the cooling plate as described for agarose isoelectric focusing. The electrode wicks were soaked in the appropriate electrode solution then blotted on filter paper for approximately 10 seconds. The anode solutions for the pH 3-10 and pH 8-10.5 intervals were 0.04M (DL) aspartic acid (L.Light and Co. Ltd., Colnbrook, England) and 0.25M N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma) respectively.

The cathode solution for both intervals was 1M NaOH. Plates were run either in the "short" direction with the electrode wicks placed along the long edges or in the "long" direction with the electrode wicks along the short edges of the gel.

The power pack was adjusted to deliver a maximum of 30W with unlimited current and voltage. Gels run

in the short direction were prefocused for 20 minutes and focused for 1¼ hours, whereas long gels were prefocused for 30 minutes and focused for 2 hours. The focusing times were based on the time required for focusing of two samples of haemoglobin applied opposite each other near each electrode. Prefocusing was carried out before samples were applied to remove free acrylic acid and establish the pH gradient, thereby decreasing the time of exposure of samples to the focusing conditions.

Preliminary work on serum proteins

pH 3-10 gradients were used and gels run in the short direction. All samples were applied near the cathode. Several methods have been described for applying samples to flat bed polyacrylamide gels including soaking the sample into small pieces of chromatography paper, on a semicircle of paper standing on the gel, in a small rectangular basin placed on the gel surface, as a droplet, streak or rectangular area on the gel surface or in a rectangular well in the gel (Vesterberg, 1975). The manufacturers recommended either application to the gel surface through rectangular holes cut in a polyester applicator strip, or soaking the sample into pieces of Whatman 3MM filter paper (Pharmacia, 1979b), while manufacturers of similar apparatus recommended Paratex application papers (LKB, 1977).

Five methods of sample application were investigated:

(a) Application to the gel surface through rectangular holes in a polyester applicator strip (Pharmacia Ltd.).

(b) Application as 10 - 20µl samples soaked into 0.5 x 1cm rectangles of Whatman 3MM filter paper and placed on the gel surface.

(c) Application as 10 - 20µl drops soaked into 0.5 x 1cm rectangles of Paratex.

(d) Application to the surface as 2µl drops using a Quantitative Microlitre Sample Dispenser (Corning Medical, Catalogue No. 470152).

(e) Application direct to the gel surface with a 1.5µl or 3µl Cellogel sample applicator (Whatman Lab-Sales Ltd.).

Application papers and strips were removed 20 minutes after the start of focusing.

Samples applied into wells in the applicator strip showed longitudinal "tailing" and a tendency to diffuse sideways underneath the strip, while the strip itself left a mark on the gel surface. Whatman 3MM filter paper and Paratex caused longitudinal "tailing". Paratex was easily removed, but filter paper stuck to the gel surface leaving a damaged area. Application to the gel surface as a drop or by means of the Cellogel applicator produced minimal "tailing"

and little or no mark at the application point. The 3µl Cellogel applicator gave similar qualitative results to the 1.5µl applicator but the application point was twice as wide (Fig. 4.20). For future work, both applicators were used. A number of problems were encountered during focusing of serum proteins.

Arcing frequently occurred from either electrode to the surface of the gel adjacent to the electrode or at the edge of the gel approximately $^{1}/_{3}$ of the distance between the anode and cathode. Arcing near the electrodes was associated with the production of water at the electrodes and was more frequently observed at the cathode. This problem was reduced, but not eliminated, by stopping focusing to blot the wicks with filter paper. Arcing at the edge of the gel was associated with thinning of the gel in this region and was reduced by placing a small strip of polyacrylamide gel over the area. Continued arcing resulted in burning of the GelBond film and eventually, damage to the teflon-covered cooling plate.

Lifting of the gel from the GelBond or glass frequently occurred near the anode, resulting in distortion of the pH gradient and the protein bands.

Both arcing and gel distortion were reduced by removal of the teflon cooling plate cover and replacement with Melanex polyester film, type S (ICI Plastics

Division, Welwyn Garden City, Herts.) on top of a thin layer of liquid paraffin, but the problem was never eliminated and resulted in destruction of many gels during focusing of serum proteins and enzymes.

Results obtained with sera from several species showed superior resolution to those obtained with agarose, particularly in the middle and cathodal end of the pH 3-10 gradient. However, unevenness near the anode distorted the bands. This is clearly demonstrated in Fig. 4.20 .

Lactate dehydrogenase

Acrylamide concentration was altered to 5.5% T and 3.8% C which is suitable for resolving protein molecules with a molecular weight of up to 500,000 daltons. This would provide a more robust gel without retarding the migration of lactate dehydrogenase which has a molecular weight of approximately 135,000 daltons (Wilkinson, 1970). Gel composition was as follows:

distilled water	- 14ml
acrylamide stock solution (20% w/v acrylamide + 0.8% w/v bisacrylamide)	- 8ml
50% (v/v) glycerol	- 6ml
TEMED	- 10µl
Pharmalyte	- 1.9ml
10% ammonium persulphate	- 100µl

pH 3-10 gradients were used and all plates were run in the short direction.

Serum samples from a variety of species were run on twelve polyacrylamide plates. In each case, bands

of enzyme activity were well resolved, straight and clearly separated. As observed in agarose gels, the multiple bands formed five main zones of activity (Fig. 4.21).

Ten plates were run with homogenates of ovine heart, kidney, liver, skeletal muscle, lung, rumen mucosa, small intestine mucosa and large intestine mucosa. More bands were visible than on agarose, each tissue showing a specific pattern. Again, bands formed five main zones of activity (Fig. 4.22).

To investigate the microheterogeneity of lactate dehydrogenase in greater detail, twelve ovine tissue homogenates were focused in a pH 3-10 gradient and the position of the sub-bands represented diagrammatically in relation to distance from the cathode and iso-electric point. All twelve tissues were focused on each of four gels.

Distance from the cathode was determined by placing the stained gel on an Ilford No. 4 darkroom lamp (Ilford Ltd., Ilford, Essex) and measuring the distance of each band from the anodal edge of the cathode wick, which was visible as a purple line across the gel. Distance was measured to the nearest 0.5mm and the bands represented diagrammatically on 1mm graph paper. pH gradients were determined by an LKB Multiphor Surface pH Electrode (LKB Produkter AB) connected to a Pye model 78 pH meter (Pye Unicam, Cambridge). The pH meter was calibrated with Buffer Solution Tablets

(Burroughs Wellcome and Co., London) of pH 4.01 and pH 9.15 at 25°C.

On completion of focusing a 1.5cm wide strip was cut from the middle of the gel. The strip was immediately placed on a graph paper template and the pH gradient measured at 5mm intervals across the gel, at room temperature. The gradient was plotted on the diagram of LDH bands. Diagrams from two of the four gels are shown in Figs. 4.23 and 4.24. The diagrams show marked discrepancies in the number, width, position and estimated isoelectric point of the bands. In addition, the pH range differs between the gels, Fig. 4.23 showing a range of 4.2 - 9.8 and Fig. 4.24 from 3.6 - 10.2 whereas the expected interval was pH 3-10.

The variation which I observed in the estimated isoelectric points may be explained by the lack of control of the temperature at which the pH gradient was measured.

However, the discrepancies between multiple band patterns in different aliquots of the same sample could not be explained. This, together with the impossibility of quantifying the bands by densitometric scanning (see page 90), caused isoelectric focusing of lactate dehydrogenase to be abandoned.

The observation that ovine lactate dehydrogenase formed five zones on isoelectric focusing is consistent

with other reports in different species. Chamoles and Karcher (1970a) using polyacrylamide rod gels, focused the five lactate dehydrogenase isoenzymes of human skeletal muscle. LDH_1 formed one main band, LDH_2 3 bands, LDH_3 one band, and LDH_4 and $_5$ at least 3 bands each. The isoenzymes appeared in the same order in the pH gradient as on electrophoresis. By using narrow pH range carrier ampholytes, they resolved LDH_4 into 7 bands. Klose and Spielmann (1975) focused pure LDH_1 and LDH_5 from mouse tissues into 1 and 5 bands respectively. Isoelectric focusing of lactate dehydrogenase in ovine serum or tissues has not been reported.

Both Chamoles and Karcher (1970a) and Klose and Spielmann (1975) reported variation in the number of bands of lactate dehydrogenase observed, but they did not comment on their significance, probably because they were not attempting to quantify the bands.

Aldolase

Initially, aldolase was focused in gels identical to those used for lactate dehydrogenase (5.5% T, 3.8% C) since aldolase is reported to have a molecular weight between 142,000 (Stellwagen and Schachman, 1962) and 160,000 daltons (Kawahara and Tanford, 1966). According to the molecular weight, aldolase should have migrated without retardation, but staining was consistently observed at the point of sample

application regardless of position in the gradient. This problem was reduced but not eliminated by applying the sample near the anode wick and reducing the total acrylamide concentration in the gel to 4.2%.

The development of the techniques for agarose and polyacrylamide isoelectric focusing of aldolase was carried out concurrently therefore the remarks regarding non-specific staining apply equally to this part of the work.

Serum samples from several species and ovine liver, heart, kidney, skeletal muscle, abomasal mucosa and rumen mucosa homogenates were run on ten plates in a pH 3-10 gradient. Serum samples showed diffuse staining, if at all, and only three gels showed distinct bands for homogenates (Fig.4.25), the results being similar to those obtained on agarose.

Problems were encountered with polymerisation of gels containing Pharmalyte 8-10.5 and it was necessary to increase the volume of TEMED from 10µl to 50µl. The same samples as above were run in each of three plates. Severe longitudinal "tailing" occurred in two plates and diffuse areas of staining in the third (Fig.4.26). In pH 3-10 gradients, skeletal muscle appears to show 5 bands, and liver one broad, major band with 2-3 minor zones (Fig. 4.25 shows bands in muscle).

For a more detailed examination of the band patterns, the six ovine tissues which showed the

greatest aldolase activity were focused in each of four gels containing Pharmalyte 3-10. The results were represented diagrammatically (Figs. 4.27 and 4.28) and the pH gradients measured. The same samples were run on a fifth gel and stained by the tetrazolium method, with fructose-1,6-diphosphate omitted from the reaction mixture.

The pattern of non-specific bands observed in this gel was compared with the band pattern in the gels stained for aldolase activity, with the substrate included in the reaction mixture to allow differentiation between non-specific activity and aldolase activity in the latter gels. As previously demonstrated for lactate dehydrogenase there were differences in the number, width, position and estimated isoelectric point of the bands. Isoelectric focusing of aldolase was abandoned since the discrepancies observed between different aliquots of the same sample made qualitative studies unreliable, while quantitation was impossible using the available equipment (see page 90).

Susor, Kochman and Rutter (1969 and 1973) resolved crystalline aldolase A from rabbit skeletal muscle into five bands in a sucrose density gradient isoelectric focusing column and reported that focusing of aldolase C from rabbit brain produced a similar result. Schapira, Gregori and Hatzfeld

(1977) have apparently published the only report of the use of flat bed polyacrylamide isoelectric focusing for aldolase when they separated aldolase B from human liver into 5 bands. Their findings differed from those of Ikehara and Endo (1970) who resolved rat liver aldolase into one broad peak on sucrose density gradient isoelectric focusing with a higher isoelectric point than the five skeletal muscle peaks. There are no previous reports of isoelectric focusing of aldolase from sheep serum or tissues.

Glucosephosphate isomerase

Apparently, the flat bed technique has not been described for focusing of this enzyme in vertebrates, while the patterns of glucosephosphate isomerase in sheep serum and tissues have not been studied by any form of isoelectric focusing.

Gels of 5.5% T and 3.8% C were used, allowing unretarded migration of glucosephosphate isomerase which has a molecular weight of about 130,000 daltons (Yoshida and Carter, 1969). (see lactate dehydrogenase isoelectric focusing for gel composition). Samples were applied near the anode wick to avoid interference due to staining at the point of application, since the bands focused near the cathode.

Serum samples from several species and homogenates of ovine heart, liver, kidney, lung, skeletal

muscle, rumen mucosa, small intestine mucosa and large intestine were focused in a gradient of pH 3-10.

Five plates were run in the short direction. All samples showed a similar pattern of five major bands with one minor band cathodal, and 2-5 minor bands anodal to the major bands (Fig. 4.29).

A further 5 plates were run in the long direction, using the same samples. The band patterns were identical to those of samples run in the short direction and although separation between bands was improved, resolution was poor, therefore no advantage was obtained over running gels in the short direction.

One plate containing Pharmalyte 8-10.5 was run but this was found to be an unsuitable gradient for glucosephosphate isomerase isoenzymes, all the bands migrating close to the anode electrode wick. Although good resolution of multiple forms of glucosephosphate isomerase was obtainable, further investigations using this technique were not undertaken because of the impossibility of quantifying the closely stacked bands by conventional densitometry. This has been discussed on page 97.

The band patterns which I observed differ from those reported in different species.

Payne, Porter and Gracy (1972) focused extracts of rat skeletal muscle, liver, kidney and brain in a sucrose density gradient. All tissues showed a

similar pattern with one major peak and 1-3 minor, more anodal peaks.

Blackburn et al. (1972), using the same technique resolved crystalline rabbit muscle glucosephosphate isomerase into one major peak with a minor peak on either side.

Ross (1977) and Wright, Southgate and Ross (1979) compared patterns of the enzyme in the fluke Schistosoma using flat bed polyacrylamide gel isoelectric focusing, and found a large number of bands which differed between species and strains of the parasite. Similarly, in vertebrates there appear to be marked differences both between species and within species (Kahn et al., 1976; Gibson et al., 1978; Charles and Lee, 1980).

Aspartate aminotransferase

Focusing of aspartate aminotransferase was conducted in gels of 7.6% T and 3.8% C since such gels are suitable for resolving proteins with a molecular weight of approximately 75,000-100,000 daltons. Aspartate aminotransferase has a molecular weight of approximately 89,000 daltons (Campos-Cavieres and Munn, 1973). Gel composition was as follows:

distilled water	- 10ml
acrylamide stock solution (20% w/v acrylamide + 0.8% w/v bisacrylamide)	- 11ml
50% glycerol	- 8ml
Pharmalyte	- 1.9ml

TEMED	-	10µl
10% ammonium persulphate	-	100µl

Samples were applied in the middle of the gradient to avoid interference of the application point with focused bands. Serum samples from a variety of species and homogenates of ovine liver, heart, kidney, lung, skeletal muscle, small intestine mucosa and large intestine mucosa were focused in pH 3-10 gradients. In three plates run in the short direction aspartate aminotransferase activity was only visible for liver, heart, kidney and skeletal muscle samples. Three bands were visible - one diffuse band near the cathode and one sharp and one diffuse band approximately two-thirds of the distance from the cathode to the anode (Fig. 4.30). When extracts of liver, heart, kidney and skeletal muscle were run in the long direction in the same gradient, the diffuse anodal band resolved into two bands to give a total of one cathodal and three anodal bands (Fig. 4.31).

Serum and lung, small intestine and large intestine homogenates consistently failed to show aspartate aminotransferase activity.

Isoelectric focusing of aspartate aminotransferase was not investigated further in view of the insensitivity of the staining technique resulting in an inability to detect the enzyme in serum samples, and the difficulties encountered when attempting to quantify the

bands due to the production of small bubbles in the agar overlay during staining. These problems have been discussed on page 99.

Campos-Cavieres and Munn (1973) used flat bed polyacrylamide gel isoelectric focusing to study the microheterogeneity of the purified cytoplasmic isoenzyme of aspartate aminotransferase from sheep liver. They reported three main bands, α, β and γ with isoelectric points of 5.75, 5.56 and 5.35 respectively. Each main band consisted of two sub-bands, less than 0.06pH units apart. Although I did not measure the isoelectric points the position of the three anodal bands in the gel (6-7cm from the cathode wick) indicated an isoelectric point of between 5 and 6.

Conclusions

Isoelectric focusing in polyacrylamide gel was considered to be unsuitable for further study for the following reasons:

(a) Results for aliquots of the same sample were inconsistent.

(b) Quantitation of bands by conventional scanning densitometry was impossible.

(c) Arcing frequently occurred for no explicable reason, which was dangerous and resulted in destruction of gels.

PART 3

DEVELOPMENT OF ELECTROPHORETIC TECHNIQUES

MATERIALS, METHODS AND RESULTS

Polyacrylamide gel electrophoresis was carried out on the FBE-3000 Flat Bed Apparatus. Conversion from an isoelectric focusing system to an electrophoresis system was achieved by replacing the isoelectric focusing lid with a plain perspex lid (Fig. 4.32). The apparatus was connected to the Chandos PSU 2500 V power pack.

This equipment was used for polyacrylamide gel electrophoresis of lactate dehydrogenase, aldolase, glucosephosphate isomerase and aspartate aminotransferase while creatine kinase isoenzymes were separated by agarose electrophoresis in the Corning Agarose Electrophoresis System (Corning Medical) (Fig. 4.33).

Preparation of plates

Gel composition was varied for different enzymes, but was based on the method of Crofton (1979). Gels consisted of an appropriate gel buffer, acrylamide stock solution (20% (w/v) acrylamide + 0.8% (w/v) bisacrylamide), TEMED, and ammonium persulphate. The latter was added immediately before pouring the plate. Deaeration of the solution was not necessary.

Plates were cast on GelBond film or glass, in the isoelectric focusing gel casting frame.

Procedure for electrophoresis

Gels were placed on the cooling plate and the circulating tap water switched on, as previously described. 250ml of the appropriate electrophoresis buffer was poured into each buffer vessel. Electrophoresis wicks consisted of four layers of Whatman No. 1 filter paper cut to the width of the gel, and 15cm long. Wicks were soaked in electrophoresis buffer, held up to drain for a few seconds then placed with one end in the buffer vessel and the other end overlapping with the edge of the gel by approximately 1.5cm. Samples were applied approximately 2cm from the edge of the cathode wick with a 1.5µl Cellogel applicator. The voltage and time for electrophoresis varied for different enzymes.

Lactate dehydrogenase

Samples of ruminant serum and ovine homogenates of heart, liver, kidney, lung, skeletal muscle, rumen mucosa, small intestine mucosa and large intestine mucosa were used during the development of the technique. All plates were run for three hours at 300V with unlimited wattage and current. The gel and electrophoresis buffers were 0.1M phosphate buffer, pH 7.5 (Kubicz and Wolanska, 1977).

Three plates were run in gels of 10.2% T and 3.8% C. The isoenzyme bands were very close together, the fastest-migrating band (LDH_1) travelling only

1.5cm from the origin. By reducing the total acrylamide concentration to 6.9%, LDH_1 migrated 3cm from the origin but the isoenzymes were only 1.2mm apart. Gels of 5.5% T and 3.8% C gave a migration distance of 4cm for LDH_1, with 5-6mm between each of the five bands observed (Fig. 4.34). These findings were in agreement with those of Dietz and Lubrano (1967) using rod gels. Gels of 5.5% T were therefore used for subsequent work, the composition being as follows:

0.1M phosphate buffer, pH 7.5	-	22ml
acrylamide stock solution	-	8ml
TEMED	-	50µl
ammonium persulphate	-	35mg

The reproducibility of the electrophoretic method, followed by tetrazolium staining and scanning densitometry as a means of quantifying lactate dehydrogenase isoenzymes was assessed by calculating the "between run" variation at different levels of enzyme activity.

The between run variations were determined by subjecting four aliquots of each of eight ovine serum samples to electrophoresis and calculating the mean lactate dehydrogenase level, standard deviation and coefficient of variation for each isoenzyme in each of the eight samples. Since five isoenzymes were present, there were 40 determinations altogether. The

40 mean values were plotted against their coefficient of variation to form a scatter diagram (Fig.4.35) which showed a curvilinear relationship between the two parameters. The data was arranged in ascending order of mean level of lactate dehydrogenase activity and the correlation coefficients calculated, one point at a time, commencing with the lowest mean level of activity until the correlation coefficient reached a maximum. A regression equation was calculated from this data. A second regression equation was calculated from the remaining data. The correlation and regression coefficents for the data used for the first and second regressions are shown in Table 4.1.

TABLE 4.1.

LINEAR REGRESSION ANALYSIS - BETWEEN RUN VARIATION OF LACTATE DEHYDROGENASE ISOENZYMES

	n	a	b	r	p	df
Regression 1	23	65.2	-1.508	-0.757	<0.001	21
Regression 2	17	13.3	-0.030	-0.380	>0.1	15

For statistical definitions, see page vi.

The correlation coefficient for the data used to form the first regression equation showed a highly significant negative correlation ($p<0.001$) between level of enzyme activity and coefficient of variation.

The correlation coefficient was not significant for the remaining data. From Fig. 4.35, the degree of variation to be expected from a range of lactate dehydrogenase levels can be determined. Thus for a lactate dehydrogenase isoenzyme with an activity of more than 30 IU/l, the technique will be accurate to within $\pm$ 20%, and for a level of over 50 IU/l, the accuracy will be within $\pm$ 12%. In normal young adult sheep $LDH_{1, 2}$ and $_3$ have values of approximately 120, 30 and 50 IU/l and only LDH_4 and $_5$ are likely to fall below 30 IU/l with values of approximately 15 and 20 IU/l, respectively, while pathological increases in serum isoenzyme levels would be measured with increasing precision. The technique was therefore considered to be sufficiently accurate for diagnostic purposes.

Aldolase

The electrophoresis buffer employed was as described by Harris and Hopkinson (1976), consisting of 0.1M Tris (hydroxymethyl) methylamine (Tris) (BDH), 0.1M maleic anhydride (Sigma), 0.01M $MgCl_2$ and 0.1M ethylenediaminetetra-acetic acid (disodium salt) (BDH). The gel buffer was a 1:9 dilution of the electrophoresis buffer.

In the absence of information on polyacrylamide gel electrophoresis of aldolase, it was decided to

use gels of a higher acrylamide concentration than for isoelectric focusing i.e. 9.0% T and 3.8% C since pore size is chosen to partially restrict migration during electrophoresis (Gordon, 1969) whereas for focusing, gels should be non-restrictive (Baumann and Chrambach, 1976). The samples used were the same as for the development of lactate dehydrogenase electrophoresis. After electrophoresis for up to 2½ hours at 300V with unlimited wattage and current, aldolase activity remained at the origin. The acrylamide concentration was reduced to 4.2% T, with the concentration of cross-linker remaining at 3.8%, in an attempt to prevent this occurring.

The gel composition was as follows:

gel buffer	- 24 ml
acrylamide stock solution	- 6 ml
TEMED	- 50 µl
ammonium persulphate	- 35 mg

Gels were run under the same conditions of voltage, wattage and current for times varying from ¾ to 3 hours. Migration distance was greater than in gels of 9% T, even after runs of ¾ hour. Staining was diffuse in all cases but aldolase in sera and homogenates each formed a zone of activity after electrophoresis for 2 hours. The length and position of these zones varied between tissues (Fig. 4.36).

The reproducibility of the electrophoretic

method was not determined since all the aldolase activity was localised in one diffuse band, but the method was considered to be suitable for further study.

Glucosephosphate isomerase

The lack of published information necessitated the complete development of an electrophoretic technique for glucosephosphate isomerase. The gel and electrophoresis buffer compositions were as described by Harris and Hopkinson (1976) for starch gel electrophoresis. The gel buffer was 0.017M Tris and 0.0023M citric acid, pH 8.0, while the electrophoresis buffer consisted of 0.25M Tris and 0.057M citric acid, pH 8.0. The samples and their application were as described for lactate dehydrogenase electrophoresis. Various combinations of acrylamide concentration, voltage and time were used.

Acrylamide concentrations of 5.5, 6.9, or 8.3% T, with 3.8% C were used. By increasing the acrylamide concentration without altering the voltage or time, the resolution was improved, but the bands were less well separated. Increasing the time for electrophoresis from 2 to 6 hours improved the separation between bands without an apparent effect on the resolution, while decreasing the voltage from 300 to 200V improved the resolution. Optimum results were obtained with gels of 5.5% T run at 200V for 5½ hours. 5.5% T gels were prepared as follows:

gel buffer	- 22ml
acrylamide stock solution	- 8ml
TEMED	- 50µl
ammonium persulphate	- 35mg

Tissue samples from Scottish Blackface sheep showed three main bands with two faint intermediate bands (Fig. 4.37) while serum samples showed only the three main bands.

The reproducibility of electrophoresis followed by scanning densitometry as a means of quantifying glucosephosphate isomerase isoenzymes was assessed by determining the between run variations at different levels of isoenzyme activity. Tetrazolium staining was used.

The between run variation of the electrophoretic technique was determined by subjecting four aliquots of each of eight ovine serum samples to electrophoresis. The means, standard deviations and coefficients of variation of each isoenzyme were calculated from the results obtained from the four aliquots of each serum sample. Since three isoenzymes were present in each sample, 24 sets of the above data were calculated. A scatter diagram was constructed (Fig. 4.38), and the two regression equations calculated as described for lactate dehydrogenase, the relationship between glucosephosphate isomerase activity and coefficient

of variation being curvlinear. The correlation coefficients for the data used for each regression are shown in Table 4.2.

TABLE 4.2.

LINEAR REGRESSION ANALYSIS - BETWEEN RUN VARIATION OF GLUCOSEPHOSPHATE ISOMERASE ISOENZYMES

	n	a	b	r	p	df
Regression 1	8	45.4	-0.568	-0.725	< 0.05	6
Regression 2	16	12.4	-0.022	-0.270	> 0.1	14

For statistical definitions, see page vi.

Thus, the data used for the first regression showed a significant negative correlation between glucose-phosphate isomerase isoenzyme activity and the between run coefficient of variation ($p < 0.05$). The data used for the second regression did not show a significant correlation between activity and coefficient of variation. From Fig. 4.38, glucosephosphate isomerase levels above approximately 40 IU/l are expected to be accurate to within $\pm$ 20% and levels above 100 IU/l to within $\pm$ 10%. It was considered that this degree of accuracy was sufficient for glucosephosphate isomerase electrophoresis to be included in future work since in adult sheep, only GPI I is likely to fall below 40 IU/l, having a value of approximately 30 IU/l.

Aspartate aminotransferase

Gel and electrophoresis buffers were prepared as described by Decker and Rau (1963). The gel buffer consisted of 0.025M boric acid and 0.01M NaOH, pH 8.9 and the electrophoresis buffer contained 0.3M boric acid and 0.06M NaOH, pH 8.2. All runs were carried out at 300V with unlimited wattage and current in gels of 7.5% T (Campos-Cavieres and Munn, 1973). The gel composition was as follows:

gel buffer	-	19.2ml
acrylamide stock solution	-	10.8ml
TEMED	-	50µl
ammonium persulphate	-	35mg

Serum samples from several species and homogenates of ovine liver, heart, kidney, lung, skeletal muscle, small intestine and large intestine were used in the development of the technique. All homogenates showed some activity remaining at the origin. One band was visible anodal to this while liver and heart samples showed a second band cathodal to the origin. When samples of liver and heart were run for 1, 2 and 3 hours, the separation between the two bands improved with increasing time. However, since bands were well separated after 2 hours, this time was considered to be adequate (Fig. 4.39). Serum samples failed to show activity, even at a total aspartate aminotransferase level of over 160 IU/l.

The reproducibility of the technique was not determined because quantitation of the results was not possible (see page 99) and electrophoresis of aspartate aminotransferase was not investigated further for this reason.

Creatine kinase

Creatine kinase isoenzymes were separated in agarose gel using a commercially prepared kit, the Corning Agarose Electrophoresis System (Corning Medical) (Fig. 4.33). The buffer used was 0.05M barbital buffer adjusted to pH 8.6 with 2N HCl (Corning Special Barbital Buffer Set Cat. No. 470182), supplied in pre-weighed vials. The agarose film (Corning Special Purpose Electrophoresis Film Cat. No. 470104) consisted of a layer of agarose approximately 1mm thick on a thin plastic backing adherent to a hard plastic cover, the gels containing 1% (w/v) agarose and 5% (w/v) sucrose in an AMP buffer, 0.03M, pH 8.6. Immediately before electrophoresis, the film was peeled from the hard plastic cover and 3µl of sample applied, 1µl at a time, into preformed wells in the agarose using the Corning Quantitative Microlitre Dispenser (Cat. No. 470152). Electrophoresis was carried out at 90 volts for 20 minutes using a Vokam 2541 Power Supply (Shandon Scientific Co. Ltd., Pound Lane, Willesden, London).

Gels were stained by the fluorescent method

described on page 100. Figs 4.40 and 4.41 show the results obtained after electrophoresis of creatine kinase in ovine serum and tissues.

The between run variation of the technique was determined as described on page 130 for lactate dehydrogenase. In the case of creatine kinase, four isoenzymes were present giving a total of 32 determinations from eight serum samples. The correlation coefficients are given in Table 4.3.

TABLE 4.3.

LINEAR REGRESSION ANALYSIS - BETWEEN RUN VARIATION OF CREATINE KINASE ISOENZYMES.

	n	a	b	r	p	df
Regression 1	13	66.1	-3.792	-0.707	<0.001	11
Regression 2	19	13.3	-0.023	-0.198	>0.1	17

For statistical definitions, see page vi.

The data used for the first regression shows a significant negative correlation between creatine kinase activity and degree of variation in its measurement, expressed as the coefficient of variation ($p < 0.001$). No significant correlation exists between these two parameters with the data used for the second regression. Thus, from Fig.4.42 creatine kinase isoenzyme bands with an activity of greater than 12 IU/l can be expected to be accurate to within $\pm$ 20%, and

values above 50 IU/l to within $\pm$ 12%. From the foregoing results, it was considered that measurement of creatine kinase isoenzymes after separation by electrophoresis was sufficiently accurate for inclusion in future work since in young adult sheep, only CK-MB_1 is likely to fall below 12 IU/l having a normal value of approximately 5 IU/l, while in pathological sera with increased creatine kinase activity, levels are liable to be much higher than this. The possible reasons for the presence of four creatine kinase isoenzymes rather than the three found in other species will be discussed in Chapter 8.

Conclusions

The lack of information on certain separation methods e.g. agarose isoelectric focusing of lactate dehydrogenase, aldolase and glucosephosphate isomerase, flat bed polyacrylamide gel isoelectric focusing of glucosephosphate isomerase and aspartate aminotransferase and polyacrylamide gel electrophoresis of aldolase and glucosephosphate isomerase, necessitated the development of these techniques from first principles. In other instances, for example the polymerisation of polyacrylamide gels using the method recommended by Pharmacia Ltd., the information available was erroneous and in addition, certain problems were encountered with faulty equipment. As a result of these factors, a considerable amount of time was spent

on this part of the work.

Certain techniques were abandoned because the problems they presented were insurmountable in the alloted time using the facilities available to me.

The methods selected for further study were as follows, the pages on which the techniques are described being given in brackets:

TABLE 4.4.

Enzyme	Separation Technique	Staining method
lactate dehydrogenase	polyacrylamide gel electrophoresis (p130)	tetrazolium (p 88)
aldolase	" " " " (p133)	fluorescent (p 95)
glucosephosphate isomerase	" " " " (p134)	tetrazolium (p 96)
creatine kinase	agarose gel electrophoresis (p138)	fluorescent (p100)

CHAPTER 5

NORMAL ISOENZYME LEVELS IN SERUM AND TISSUES - REVIEW OF THE LITERATURE.

INTRODUCTION

The activity of an enzyme is determined by measuring the turnover of the substrate in a specified time. Early workers used a variety of different reaction conditions for enzyme assays, and important parameters such as temperature were often uncontrolled. In 1961, the Commission on Enzymes of the International Union of Biochemistry attempted to standardise assays by recommending the use of a standard, international unit for all enzymes, which they defined as follows:

> "One unit (U) of any enzyme is that amount which will catalyse the transformation of one micromole of the substrate per minute under standard conditions... With regard to the conditions of measurements, the temperature should be stated, and it is suggested that where practicable, it should be 25°C.... The other conditions should, wherever possible, be optimal, especially with regard to pH and substrate concentration".

In 1965, a revised edition of the report suggested that the standard temperature should be changed to 30°C, but many workers continued to carry out enzyme assays at 25°C and on occasions at 37°C.

Enzyme activities in the body fluids are expressed in international units per litre (IU/l). Enzyme activity in tissues is usually expressed in IU/g wet weight of tissue or occasionally in IU/g protein, while in haemolysates, levels are expressed in IU/g of haemoglobin.

In view of the difficulty in comparing values obtained by earlier methods with those using the international unit, total enzyme levels will not be quoted unless they are in international units or their equivalent. All tables are presented in Appendix 2.

LACTATE DEHYDROGENASE

Following the observation that lactate dehydrogenase isoenzymes were present in human serum and tissues (Vesell and Bearn, 1957; Plagemann, Gregory and Wróblewski, 1960), the total enzyme level and isoenzyme distribution has been studied in the serum and tissues of numerous species including man, rats, mice, dogs, pigs, horses and cattle. The results obtained in these species are presented in Tables 5.1 - 5.6, and the reference given in each case.

The half-life times of lactate dehydrogenase in the serum have been estimated in rabbits (Amelung, 1960), man (Mattenheimer, 1971) and pigs (Bogin, Sommer and Tureck, 1977). Their results are shown in Table 5.19.

Lactate dehydrogenase isoenzymes in sheep tissues were first demonstrated by Markert and Møller (1959) who resolved sheep heart homogenates into two anodally migrating fractions on starch gel electrophoresis. Three years later, Boyd (1962) determined the normal serum and tissue total lactate dehydrogenase activity

in the sheep. His results were expressed in arbitrary units, but the highest level of activity was found in skeletal muscle, followed by cardiac muscle, kidney and liver with appreciable quantities in brain, small intestine, lung and spleen.

Boyd (1964) first quantified the isoenzymes in a number of sheep tissues by densitometry after electrophoresis in agar gel. The isoenzymes were expressed as peak areas on the densitometer tracing and have been converted to percentage values for inclusion in Table 5.8. The isoenzyme distribution in liver, heart and kidney were remarkably similar with intense LDH_1 activity and faint LDH_2 and $_3$ activity, while LDH_4 and $_5$ were usually undetectable. Marked differences were observed in the isoenzyme pattern of different skeletal muscles. Most showed intense LDH_5 activity while in diaphragm and tongue LDH_1 and $_2$ were marked, although they were not predominant. The masseter muscle however, showed intense activity of LDH_1, $_2$ and $_3$ with very little LDH_4 and $_5$.

Although sheep muscles are not distinguishable macroscopically into red and white muscle, diaphragm, tongue and masseter muscle behave as red muscle according to their lactate dehydrogenase isoenzyme distribution which would favour continuous aerobic metabolism. Muscles such as triceps and gastrocnemius behave as white muscle with a predominance of LDH_4 and $_5$, favouring intermittent anaerobic metabolic activity.

Paulson, Pope and Baumann (1966) determined the normal isoenzyme distribution in ovine tissues by the same electrophoretic method as Boyd (1964).

Boyd (1967) calculated the half-life times of LDH_1 and LDH_5 in the plasma of sheep by intravenous injection of purified isoenzyme from sheep skeletal muscle and heart. In both cases, the disappearance rates from the plasma followed a biphasic, exponential course. The first exponential phase was considered to be due to distribution of the isoenzyme between the intravascular and extravascular compartments, while the second phase was due to its actual removal or inactivation and was therefore a measure of the turnover rate of the isoenzyme. The half-lives for the two phases were found to be 2.0 and 8.0 hours for LDH_5 and 2.4 and 48.0 hours for LDH_1. The author considered that in general, the reticulo-endothelial system was involved in removal of enzymes from the circulation. He proposed that the two isoenzymes differed in that only LDH_5 is irreversibly removed from the plasma by the reticulo-endothelial system accounting for the difference in half-life times during the second exponential phase. On this basis he calculated that LDH_5 must leak from tissues at a rate fifteen times greater than LDH_1 to maintain the same level in the plasma. This is consistent with the observation that LDH_5 activity in normal

plasma is lower than that of LDH_1, despite the overall predominance of the former in the body.

In 1970, Tollersrud determined the percentage distribution of lactate dehydrogenase in Norwegian short-tailed lambs and ewes. Serum isoenzyme levels were estimated in both ewes and lambs, while tissue levels were only carried out in ewes. Total enzyme levels were expressed in arbitrary units, and 10-day old lambs showed a higher serum level than ewes. In liver extracts, Tollersrud observed that LDH_5 showed an exceptionally high cathodic electrophoretic mobility in agar gel. This phenomenon has not been reported by other workers.

Tollersrud and Baustad (1970) followed the changing isoenzyme profile in the serum of lambs from birth to 10 days old. Over the period of study the total enzyme level increased during the first day to a peak at 6-12 hours after birth with a subsequent decline to below the level found at birth, by 32-48 hours.

The authors mentioned several possible mechanisms producing the increased serum enzyme level after birth. Hormones such as adrenocorticotrophic hormone, cortisone, insulin and catecholamines increase cell membrane permeability and therefore increase serum enzyme levels, while "stress" caused by transfer from a warm to a cold environment at birth, increased physical activity and rapid growth might also be

contributary factors.

The isoenzyme distribution, however, showed only slight changes with little trend towards the adult pattern characterised by a higher LDH_1 percentage (Tollersrud, Baustad and Flatlandsmo, 1971). The increase in total serum lactate dehydrogenase after birth in lambs has also been demonstrated by Kleih and Bostedt (1974).

The normal total lactate dehydrogenase levels in international units have been estimated in lamb plasma (Gardner, 1973), and serum and tissues from yearling sheep (Keller, 1973). Keller determined the relative distribution of lactate dehydrogenase, the tissue with the highest level of activity (myocardium) being given a value of 100%. Gluteus muscle showed the second highest level of activity, followed by kidney cortex, brain and liver. Healy and Falk (1974) measured total lactate dehydrogenase activity in Merino lambs, ewes and rams. In pregnant ewes, serum lactate dehydrogenase increased from a mean of 67 IU/l (at an assay temperature of 25°C) to 85 IU/l within 24 hours after lambing ($p < 0.001$). Mean activity remained within 80-95 IU/l for the first 3 weeks of lactation. The total enzyme level was higher in lambs than ewes.

Healy and McInnes (1975) measured total serum lactate dehydrogenase in Merino and Merino cross lambs at approximately nine months of age. Four groups of

lambs received different amounts of the same diet to produce a differential rate of growth. However, no significant difference in serum lactate dehydrogenase level was observed between groups, lambs with a mean change in liveweight of +205, +163, +37 or -46g/day having mean levels of 91, 95, 88 and 77 IU/l at 25°C.

Michálek and Marcanik (1975) determined the normal isoenzyme distribution in the serum of several domestic species including sheep while Márquez et al. (1977) measured total serum and tissue levels. The values for tissues obtained by Márquez et al. were not expressed in international units and are therefore not included in Table 5.8. Boehringer (1979) have quoted the normal serum level in sheep as 530 IU/l at 25°C using a spectrophotometric assay.

The most recent publication on lactate dehydrogenase isoenzymes in the sheep appears to be that of Briand et al. (1981). Total enzyme level and isoenzyme distribution in the heart and twelve different skeletal muscles was measured. Isoenzymes were separated by polyacrylamide gel electrophoresis. The muscles were most clearly differentiated by their proportions of LDH_1, LDH_4 and LDH_5. The findings were consistant with the existence of three metabolic types of muscle:

(a) slow, mainly aerobic red muscles e.g. supraspinatus, with a low percentage of M subunits and low total lactate dehydrogenase activity.

(b) fast red muscles e.g. longissimus dorsi, with high oxidative and glycolytic capacities as indicated by a high M subunit percentage and high total enzyme activity.

(c) fast, mainly glycolytic white muscles e.g. tensor fascia lata with a high M subunit percentage and high total enzyme activity. Although LDH_5 seems to be important in glycolysis, LDH_1, in contrast to the views cited by Boyd (1964), is not always indicative of the oxidative capacity of the tissue, since it is low in fast, red muscles, in spite of their high oxidative capacity.

The total lactate dehydrogenase levels and isoenzyme distributions in the sheep, as determined by some of the above authors are presented in Tables 5.7 and 5.8.

ALDOLASE

Studies on aldolase isoenzymes in man and lower animals have been mainly qualitative in nature. Thus, the aldolase isoenzyme patterns have been described but not quantified in the tissues of man (Arnstall, Lapp and Trujillo, 1966; Pietruszko and Baron, 1967; Schapira and Nordmann, 1969; Lebherz and Rutter, 1969),

rabbits (Blostein and Rutter, 1963; Penhoet, Rajkumar and Rutter, 1966; Schapira, Dreyfus and Allard, 1968; Lebherz and Rutter, 1969), rats (Arnstall, Lapp and Trujillo, 1966; Pietruszko and Baron, 1967; Lebherz and Rutter, 1969; Kawachi et al., 1973; Guguen-Guillouzo and Hatzfeld, 1980), pigs (Lebherz and Rutter, 1969) and cattle (Blostein and Rutter, 1963; Lebherz and Rutter, 1969; Sheedy and Masters, 1969; Cardenas, Richards and Gabourel, 1978).

Quantitation of total aldolase has been reported in the serum of man, pigs and cattle and in the tissues of man, horses and cattle. Mattenheimer (1971) quoted the serum half-life of aldolase in man as 21 ± 2 hours.

Very few reports exist on the quantitation of aldolase isoenzymes in mammals, but such studies have been undertaken in human serum and tissues. Some of the quantitative results obtained in man and the domestic animals are presented in Tables 5.9 and 5.10.

In 1965, Young et al. investigated the effect of dietary and environmental factors on serum enzyme levels in normal sheep. Total aldolase activity was measured spectrophotometrically at an unspecified temperature, and was expressed in arbitrary units. Serum aldolase was found to be lowest after an extended period on pasture, but the authors could not explain

the reason for this change.

Sheedy and Masters (1969) have apparently published the only report on aldolase isoenzymes in sheep. Starch gel electrophoresis of ovine tissues produced similar results to those reported for cattle, in the same paper.

The five isoenzymes corresponding to aldolase A and C and the three hybrids formed from these two isoenzymes (A_3C_1, A_2C_2 and A_1C_3) were present in skeletal muscle, heart, lung, spleen, brain, kidney and liver. In addition, kidney and liver contained the five isoenzymes of the A-B hybrid set. Since aldolase A is common to A-C and A-B hybrid sets, a total of nine isoenzymes was observed in these two organs. B-C hybrids are apparently only formed *in vitro* (Penhoet *et al.*, 1966).

Pemberton *et al.* (1971) determined the total plasma aldolase level in 14 normal sheep. The values were estimated by a colorimetric assay and expressed in arbitrary units.

Márquez *et al.* (1977) determined the normal total serum aldolase in 20 adult sheep and obtained a value of 5.9 ± 1.9 IU/l at 37°C (range 2.9 - 10.2 IU/l).

Quantitative studies on aldolase isoenzymes in ovine tissues have not been reported, while ovine serum aldolase isoenzymes have been investigated neither qualitatively nor quantitatively.

GLUCOSEPHOSPHATE ISOMERASE

Glucosephosphate isomerase activity was first demonstrated in human serum by Bodansky (1954). In 1966, Schwartz and Bodansky showed that the enzyme migrated as a single band in the gamma-globulin region when subjected to starch gel electrophoresis.

The majority of studies involving glucosephosphate isomerase have described its allelozymes, usually in haemolysates. These genetic variants have been described in man (Detter et al., 1968; Fitch, Parr and Welch, 1968; Harris and Hopkinson, 1976; Kahn et al., 1976; Gibson et al., 1978; Satoh and Mohrenweiser, 1979), monkeys (VandeBerg and Stone, 1978), mice (Carter and Parr, 1967; Peterson, Friar and Wong, 1978; Charles and Lee, 1980), rabbits (Welch, Fitch and Parr, 1970), cats (Auer and Bell, 1980), pigs (Saison and O'Reilly, 1971; Jørgensen et al., 1976; Rasmusen, Beece and Christian, 1980) and horses (Sandberg, 1973).

Total glucosephosphate isomerase levels have been quantified in human serum, heart and red blood cells, rabbit, canine, feline, equine and bovine red blood cells, canine heart and liver and pre-ruminant and ruminant calf liver. The results obtained in these species are shown in Table 5.11.

Yoshida and Carter (1969) have quantified the multiple forms of crystalline rabbit muscle glucose-

phosphate isomerase, while Blackburn et al. (1972) quantified the multiple forms of the enzyme in both crystalline and crude muscle extracts from the rabbit. Glucosephosphate isomerase isoenzymes do not appear to have been quantified in the other domestic species.

In the sheep, Yoshida and Carter (1969) have noted that genetic variants occur, but their results have not been published. The starch gel electrophoretic patterns were said to be similar to those which they reported in rabbit haemolysates, with two main areas of activity and a third band which was considered to be derived from one of the other two by oxidation of its disulphide bonds.

Smith and Lee (1974) measured total glucosephosphate isomerase in ovine erythrocytes by spectrophotometric assay and obtained a value of 17.8 ± 2.74 IU/g Hb at 25°C. The mean values in ruminants were lower than in the other species investigated. (Table 5.11).

Baker and Manwell (1977), using starch and acrylamide gel electrophoresis found glucosephosphate isomerase to be monomorphic in the liver and kidney of 47 Merinos and Merino crosses.

Le Riche and Sewell (1978), in a study of the patterns of glucosephosphate isomerase in Echinococcus granulosus cysts in sheep liver, observed five anodally migrating bands of glucosephosphate isomerase from host tissue on starch gel electrophoresis but the

breed of sheep was not stated.

Glucosephosphate isomerase isoenzymes have not been quantified in sheep serum or tissues.

CREATINE KINASE

Comparisons between the results obtained by different authors are complicated by the large number of different methods used for total creatine kinase assays.

Early creatine kinase assays were colorimetric (Kuby, Noda and Lardy, 1954) and relatively insensitive, but the development of an enzyme-coupled spectrophotometric assay by Oliver (1955) increased the sensitivity by a factor of 3 or 4 (Rotthauwe and Cerqueiro-Rodriguez, 1964). The method described by Oliver depends on the same series of reactions as the creatine kinase staining techniques described on page 100 and is often erroneously referred to as an "activated" method. In 1967, Rosalki modified the method of Oliver by including either cysteine or glutathione as enzyme activators, thereby increasing the sensitivity of the technique approximately 10-fold. Most "activated" techniques now used for creatine kinase assays (e.g. Boehringer, 1979) are based on the original method of Rosalki, but "activated" colorimetric methods have also been described (Smith and Healy, 1968).

In 1961, Hughes established the normal total

enzyme level in human serum in arbitrary units, but it was not until the mid 1960's that the isoenzymes were described in human tissues (Deul and van Breeman, 1964; Burger, Richterich and Aebi, 1964; Rosalki, 1965).

In the domesticated animals, the majority of papers report the total creatine kinase levels in serum and tissues in species including dogs, cats, pigs, horses and cattle. The isoenzymes of creatine kinase have been quantified in the serum and tissues of man, rats, mice, dogs, pigs, and horses. The results obtained are presented in Tables 5.12 - 5.17. Where the information was available, the type of assay used (activated or non-activated, and colorimetric or spectrophotometric) is indicated for each author.

The serum half-life times of creatine kinase have been estimated in man, dogs, pigs, horses and calves and the results are shown in Table 5.19.

The total creatine kinase levels in ovine tissues were first reported by Brown and Wagner (1968) in an adult Merino. The values obtained were expressed in Sigma units/100mg wet weight of tissue. When the values for each tissue are expressed as a percentage of the level in the tissue with the greatest activity (skeletal muscle) the results are as follows - rectus abdominis muscle (100%), cerebrum (43.2%), spinal cord (29.1%), omasum (27.0%), reticulum (24.0%),

rumen (21.1%), cerebellum (19.7%), duodenum (15.6%), rectum (15.6%), adrenal gland (13.7%), spleen (13.5%), myocardium (13.3%), abomasum (13.3%), abdominal aorta (8.9%), urinary bladder (8.9%), kidney (4.3%), pancreas (3.9%), lung (3.9%), distal colon (2.7%), lymph node (1.4%) and liver (0.7%). The authors considered that increased serum creatine kinase levels in the sheep would most likely be the result of skeletal, smooth or heart muscle damage, while elevations could possibly result from inflammatory or degenerative conditions of the nervous tissue, spleen and adrenal glands. Such conditions affecting the liver, kidneys, lungs, pancreas and lymph nodes would be unlikely to increase serum creatine kinase levels.

Smith and Healy (1968) reported the normal total serum creatine kinase level as 13.0 $\pm$ 8.0 IU/l in ten Merino sheep sampled daily for five days. The assay was an "activated" method based on the colorimetric method of Kuby, Noda and Lardy (1954).

Tollersrud (1971) determined the normal total enzyme level in the serum of Norwegian short-tailed sheep, but his results were expressed in Sigma units.

Healy and Falk (1974) estimated the normal total serum enzyme level in Merino lambs at various intervals after birth and in a Merino flock consisting of ten rams, 4-16 months old and ten ewes, 4 months to 6½ years old. Creatine kinase was assayed by the

method of Smith and Healy (1968). The results are presented in Table 5.18. Pregnancy, lactation, shearing, fasting and water deprivation did not affect the serum creatine kinase level, but transportation for 60 hours produced a significant elevation, with a return to the normal level within three days.

Bostedt (1976) reported the normal total serum creatine kinase level to be 23.4 $\pm$ 18.9 IU/l in 88 ten day old, Merino-cross lambs, using the method of Oliver (1955) at an unstated temperature, while Boyd (1976) used the Boehringer "activated" method to determine the normal total creatine kinase level in the tissues and plasma of ten lambs. The half-life of the enzyme was also determined by following the plasma levels after the intravenous injection of a crude ovine muscle homogenate. The enzyme activity showed an exponential decline, with a plasma half-life of 62 minutes.

The total creatine kinase distribution in ovine tissues was determined by Garouachi et al. (1978), who expressed the values for each tissue as a percentage of the activity in the tissue with the highest level of the enzyme. Skeletal muscle showed the greatest activity, followed by myocardium (19.3%), brain (8.0%), lung (7.9%), thyroid (5.0%), uterus and liver (1.0%), rumen (0.5%) and spleen and

kidney (0.1%). The order of activity differs somewhat from that of Brown and Wagner (1968) but confirms the high level of activity in muscle and nervous tissue and the low level in the parenchymatous organs.

The normal level in ovine serum reported by Boehringer (1979) was 21 IU/l using their "activated" method at 25°C while Boss, Gerber and Tschudi (1979) reported the normal level as 49 $\pm$ 26 IU/l in adult sheep of several Swiss breeds, using the same technique. The level was shown to be independent of age, breed and sex.

Gooneratne and Howell (1980) also using the method of Boehringer (1979) found the normal total creatine kinase level to be 16.3 $\pm$ 6.10 IU/l in plasma and 6.5 $\pm$ 5.99 IU/l in cerebrospinal fluid in twelve, 9-12 month old Merino wethers. These authors appear to have published the only report of creatine kinase isoenzymes in the sheep but their observations were purely qualitative and were confined to plasma samples from three sheep with chronic copper poisoning.

The total creatine kinase levels in the sheep reported by some of these authors are given in Table 5.18. The normal serum and tissue isoenzyme levels have not been reported in this species.

CONCLUSIONS

The normal absolute level and percentage distribution of the isoenzymes of lactate dehydrogenase have been fairly well documented in the serum and tissues of man, dogs, horses and cattle, in the tissues of laboratory animals and in the serum of pigs and sheep. Quantitative isoenzyme studies in ovine tissues with the results expressed in international units appear to be lacking, although there are several reports on the lactate dehydrogenase isoenzyme percentages in the tissues of this species. The percentage distribution in the tissues of sheep is essentially similar to that of cattle but differs markedly from the patterns observed in other species, while the serum isoenzyme distribution is unique to each species. In adult cattle for example, LDH_1 shows the greatest activity followed by LDH_2, LDH_3, LDH_4 and LDH_5 whereas in adult sheep, the order is $LDH_1 > LDH_3 > LDH_2 > LDH_4 > LDH_5$.

There are few reports on the quantitation of aldolase isoenzymes in vertebrates, and none in the sheep, while glucosephosphate isomerase isoenzymes do not appear to have been quantified in the domestic species other than laboratory animals.

The creatine kinase isoenzyme distribution in the serum and tissues of man and the tissues of laboratory animals have been described by several authors

but in the case of dogs, pigs and horses, apparently only one such report exists for each species, while the creatine kinase isoenzyme distribution in ruminants has not been investigated.

CHAPTER 6

THE DETERMINATION OF ISOENZYME LEVELS IN NORMAL SHEEP

PART 1 - THE EFFECT OF STORAGE ON ENZYME AND ISOENZYME ESTIMATIONS

A. THE EFFECT OF STORAGE AT -70°C ON TOTAL ENZYME LEVELS

INTRODUCTION

Since it would be impossible to analyse all samples on the day of collection, it was decided that samples should be frozen at -70°C on dry ice. Significant loss of lactate dehydrogenase activity in sheep serum is known to occur within five days at -20°C (Tollersrud, 1969), whereas lactate dehydrogenase and creatine kinase, at least in human serum, are reported to be stable for up to six years at -70°C (Yasmineh et al., 1978). The effect of storage at -70°C on sheep serum lactate dehydrogenase, glucosephosphate isomerase, creatine kinase and aldolase does not appear to have been investigated.

MATERIALS AND METHODS

Venous blood samples were taken from seven, clinically normal sheep of various breeds into 7ml, plain Vacutainer tubes (Becton-Dickinson U.K. Ltd., York House, Empire Way, Wembley) using 20g x 1½" Vacutainer needles (Becton-Dickinson U.K. Ltd.). The samples were allowed to clot at room temperature, and the serum was harvested after centrifugation for 5

minutes at 3000rpm. Each sample was divided into two aliquots which were placed in 75 x 12 mm polypropylene tubes (Hughes and Hughes Ltd., Romford, Essex).

Enzyme estimations were carried out in a Cecil CE 292 Digital Ultraviolet Spectrophotometer (Cecil Instruments Ltd., Milton Industrial Estate, Cambridge) connected to a TE-7 Tempette circulating water bath (MacKay and Lynn Ltd., Edinburgh). Lactate dehydrogenase was assayed by the method of Calbiochem-Behring (LDH-L-S.V.R., Calbiochem-Behring Corporation, 10933 North Torrey Pines Road, La Jolla, California, U.S.A.), glucosephosphate isomerase by the method of Sigma (No. 355-UV, Sigma Ltd.), creatine kinase by the glutathione "activated" method of Sigma (No. 45-UV, Sigma Ltd.) and aldolase using the Boehringer Test - Combination (Boehringer Mannheim GmbH). All four methods were spectrophotometric. Serum for creatine kinase assays contained 2µl of a 1.0M solution of the reducing agent dithiothreitol (Sigma Ltd.) per 100µl of serum (final concentration 20mM). The effects of dithiothreitol are discussed on page 175. Enzymes were assayed at 30°C with the exception of aldolase which was measured at 37°C. The accuracy was monitored by means of quality control sera (Sigma Enzyme Control 2-N, Sigma Ltd. and Precinorm E, Boehringer Mannheim GmbH).

One aliquot of each sample was assayed within two hours of collection while the second aliquot was kept at -70°C for 8 weeks after which it was allowed to thaw at room temperature for enzyme analysis.

RESULTS

TABLE 6.1

TOTAL SERUM ENZYME LEVELS - COMPARISON BETWEEN SAMPLE LEVELS BEFORE AND AFTER STORAGE BY PAIRED t-TESTS

df=6

Enzyme	day	n	$\bar{x}$ IU/l	s	t
Lactate dehydrogenase	0	7	465.3	320.30	1.584
	56	7	438.6	293.14	
Glucosephosphate isomerase	0	7	636.8	266.98	-4.350**
	56	7	663.8	261.94	
Creatine kinase	0	7	101.1	42.32	1.258
	56	7	96.9	41.83	
Aldolase	0	7	111.7	78.29	1.797
	56	7	102.1	79.48	

For statistical definitions, see page vi.

The mean value and standard deviation was calculated for each enzyme before and after storage for 8 weeks and paired t-tests were carried out on the data for each enzyme to compare the corresponding individual values before and after storage. The results are shown in Table 6.1.

No significant change in activity with storage at -70°C was observed for lactate dehydrogenase, creatine kinase or aldolase, while glucosephosphate isomerase showed a significant increase in activity after storage ($p < 0.01$). The mean increase in glucosephosphate isomerase activity was 4.2% as calculated from $\frac{\bar{\Delta}}{\bar{x}(\text{day } 0)} \times 100$.

DISCUSSION

The reason for the increase in glucosephosphate isomerase activity with storage was not apparent but has been reported for bovine, ovine and porcine serum aspartate aminotransferase stored at room temperature and for bovine serum alanine aminotransferase stored at room temperature and at 4°C (Tollersrud, 1969). Tollersrud considered that the increase in amino-transferase activity at room temperature might be associated with bacterial growth since the sera became turbid, but he could not explain the increase in activity at 4°C. Since bacterial growth could not have contributed to the increased glucosephosphate isomerase activity at -70°C, it is possible that there was a loss of activity in the fresh sera caused by the delay of approximately 2 hours between coll-ection and analysis, whereas stored samples were placed in dry ice immediately after harvesting of

the serum, and were analysed immediately after thawing.

Although the change in glucosephosphate isomerase activity with storage was statistically significant, it represented a mean increase of only 4.2% (range 1.1 - 10.9%). This small increase would be of little practical consequence but, for the purposes of this work, it was considered that all samples for glucosephosphate isomerase estimation should be stored for an equal length of time at -70°C, and samples for lactate dehydrogenase, glucosephosphate isomerase, creatine kinase or aldolase assays should be kept at -70°C for a maximum of 8 weeks, as the effect of storage for longer periods was not investigated.

B. THE EFFECT OF STORAGE AT -70°C AND DITHIOTHREITOL ON CREATINE KINASE ISOENZYMES

INTRODUCTION

Creatine kinase is known to be a very labile enzyme, requiring protection from heat and oxidation. Two distinct types of inactivation occur - an initial rapid inactivation which can be reversed by the addition of thiol compounds such as dithiothreitol and a slower, irreversible inactivation. The former is considered to be due to oxidative and the second to thermal effects (Morin, 1977).

The isoenzymes of creatine kinase show differences in stability, and Tsung (1976) in an extensive study of creatine kinase isoenzymes in human tissues considered BB to be the most stable isoenzyme, followed by MM and MB, while other authors (Szasz, Gerhardt and Gruber, 1978) have reported the MM isoenzyme to be the most stable.

The aim of this investigation was to evaluate the effect of freezing in the presence or absence of dithiothreitol on serum and tissue creatine kinase isoenzymes and to determine whether the addition of dithiothreitol either before freezing or after thawing affected the isoenzyme levels. In tissue homogenates, the effect of ageing for 24 hours at 4°C was assessed since there would inevitably be some delay between sample collection and freezing at -70°C because of the time required to prepare the homogenates.

In view of the reported lability of the MB isoenzyme, human serum from patients with myocardial infarction containing increased levels of this isoenzyme was used since a preliminary study had indicated that MB constituted a small percentage of ovine serum creatine kinase, while for tissue isoenzyme studies, ovine heart homogenates were used.

SERUM

MATERIALS AND METHODS

Serum samples from eight patients with myocardial infarction were supplied by the Clinical Chemistry Department, Edinburgh Royal Infirmary.

Each sample was divided into five 200µl aliquots which were placed into 0.5ml polypropylene micro-centrifuge tubes (Hughes and Hughes Ltd.). The five aliquots were treated as follows:

TABLE 6.2

EXPERIMENTAL DESIGN

Treatment	n	Temperature of storage	dithiothreitol	remarks
1	8	4°C	-	
2	8	4°C	+	
3	8	-70°C	-	
4	8	-70°C	+	dithiothreitol added before freezing
5	8	-70°C	+	dithiothreitol added after thawing

Aliquots for treatments 1 and 2 were kept at 4°C until analysed. The unfrozen aliquots were analysed on the day of collection whereas the frozen aliquots were kept overnight at -70°C. Aliquots

for treatments 3-5 were placed in dry ice at the same time as those for treatments 1 and 2 were assayed for total creatine kinase. 4µl of 1M dithiothreitol/200µl serum was added to aliquots 2, 4 and 5, giving a final concentration of 20mM dithiothreitol.

Total creatine kinase assays were carried out using the Sigma 45-UV spectrophotometric assay at 30°C and creatine kinase isoenzymes were separated and quantified as described in Chapter 4.

RESULTS

TABLE 6.3.

SERUM CREATINE KINASE ISOENZYME LEVELS - EFFECTS OF STORAGE AND DITHIOTHREITOL.

		TOTAL CK(IU/l)		MM (IU/l)		MB (IU/l)		BB(IU/l)	
Treat-ment	n	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
1	8	1126.9	195.03	989.1	170.70	137.8	41.11	0	-
2	8	1168.0	180.08	985.6	164.59	182.4	59.37	0	-
3	8	1090.0	220.95	913.6	171.37	176.3	72.45	0	-
4	8	1143.7	186.95	940.8	157.77	202.9	69.80	0	-
5	8	1128.0	174.23	934.2	144.12	193.8	59.87	0	-

For statistical definitions, see page vi.

The mean value and standard deviation of the total creatine kinase and isoenzyme levels were

calculated for each sample treatment, and the results are presented in Table 6.3.

TABLE 6.4.

SERUM CREATINE KINASE AND ITS ISOENZYMES IN IU/l - COMPARISON BETWEEN SAMPLE TREATMENTS BY PAIRED t-TESTS

n = 8 for each treatment; df = 7

Treatments Compared	TOTAL CK	MM	MB
	t	t	t
1/2	-2.013	0.182	-3.289 *
1/3	1.322	2.290	-2.383 *
1/4	-0.396	0.877	-3.571 **
1/5	-0.029	1.246	-3.959 **
2/3	2.570*	2.022	0.264
2/4	0.739	0.862	-0.799
2/5	1.059	1.163	-0.601
3/4	-1.623	-0.791	-2.837 *
3/5	-1.159	-0.749	-1.408
4/5	0.625	0.268	-0.955

For statistical definitions, see page vi.

Paired t-tests were carried out to compare the total creatine kinase and isoenzyme levels in fresh and frozen serum in the presence or absence of dithiothreitol. Each sample treatment was compared with all other treatments. The results are shown in Table 6.4.

The total creatine kinase level was significantly higher in fresh samples in the presence of the reducing agent dithiothreitol (treatment 2) than in frozen samples in the absence of dithiothreitol (treatment 3), ($t = 2.570$; $p < 0.05$). Differences in total creatine kinase between other sample treatments were not statistically significant. The level of the MM isoenzyme was not significantly affected by freezing or the reducing agent. In the case of the MB isoenzyme, fresh samples in the absence of dithiothreitol (treatment 1) contained a significantly lower MB activity than all other methods of treatment, while samples kept at -70°C in the absence of reducing agent showed significantly lower MB levels than those at -70°C in the presence of the reducing agent. No significant difference was observed between frozen samples to which dithiothreitol had been added before freezing and those to which it had been added after thawing.

HEART HOMOGENATES

MATERIALS AND METHODS

Approximately 5g of myocardium was removed from the left ventricle of eight, clinically normal, Scottish Halfbred lambs immediately after death by exsanguination. Samples were kept on ice in a polystyrene

container prior to homogenisation (for preparation of homogenates, see page 180.

Five, 200µl aliquots of the supernatant obtained after centrifugation of each heart homogenate were put into 0.5ml polypropylene microcentrifuge tubes. Protein determinations were carried out by the biuret method on 100µl sample of the remaining supernatants (for details of protein determinations, see pages 181 and 182).

TABLE 6.5.

EXPERIMENTAL DESIGN.

Treat-ment	n	Temperature of storage	dithiothreitol	remarks
1	8	4°C	-	
2	8	4°C	+	
3	8	-70°C	-	
4	8	-70°C	+	dithiothreitol added before freezing
5	8	-70°C	+	dithiothreitol added after freezing

Dithiothreitol was added to aliquots 2, 4 and 5 to a final concentration of 20mM. Aliquots 1 and 2 were kept at 4°C for 24 hours before analysis whereas aliquots 3, 4 and 5 were placed on dry ice immediately after preparation. The total creatine kinase assays and isoenzyme quantitation were as described

for the effect of storage on serum creatine kinase. Results were expressed as IU/g protein.

RESULTS

TABLE 6.6.

CREATINE KINASE ISOENZYME LEVELS IN HEART HOMOGENATES - EFFECTS OF STORAGE AND DITHIOTHREITOL.

Treatments	n	TOTAL CK (IU/g protein)		MM (IU/g protein)		MB (IU/g protein)		BB (IU/g protein)	
		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
1	8	18172.9	4796.37	18172.9	4796.37	0	-	0	-
2	8	25700.4	8217.50	25700.4	8217.50	0	-	0	-
3	8	18700.4	5312.11	18700.4	5312.11	0	-	0	-
4	8	28531.0	6555.25	21489.4	4818.34	4341.5	3872.71	0	-
5	8	16873.6	6078.79	16873.6	6078.79	0	-	0	-

For statistical definitions, see page vi.

The mean and standard deviation were calculated for the level of total creatine kinase and its isoenzymes for each sample treatment. The results are given in Table 6.6.

TABLE 6.7.

CREATINE KINASE AND CK-MM IN IU/g PROTEIN IN HEART HOMOGENATES - COMPARISON BETWEEN SAMPLE TREATMENTS BY BY PAIRED t-TESTS.

n = 8 for each treatment; df = 7

Treatments Compared	Total CK	MM
	t	t
1/2	-3.189*	-
1/3	-0.195	-
1/4	-3.908**	-1.664
1/5	0.507	-
2/3	2.873*	-
2/4	-0.076	1.516
2/5	3.826**	-
3/4	-2.708*	-1.077
3/5	0.966	-
4/5	5.601***	2.864*

For statistical definitions, see page vi.

Paired t-tests were carried out to compare the total creatine kinase level of corresponding samples, subjected to the conditions indicated in Table 6.5.

The level of the MM isoenzyme in samples of the treatment 4 group were compared with the corresponding values for MM in all other treatment groups. The results of the paired t-tests are shown in Table 6.7.

Total creatine kinase in samples aged for 24 hours at 4°C was significantly higher in those samples to which dithiothreitol had been added (treatment 2) than in those stored in the absence of the reducing agent (treatment 1) ($p < 0.02$). Similarly samples kept at -70°C in the presence of dithiothreitol (treatment 4) showed significantly higher total enzyme levels than those without dithiothreitol (treatment 3) ($p < 0.05$). Treatment 2 samples also showed significantly higher total creatine kinase levels than treatment 3, samples at -70°C in the absence of dithiothreitol ($p < 0.05$) and treatment 5, samples at -70°C with the addition of dithiothreitol after thawing ($p < 0.01$). A highly significant difference was observed in the total creatine kinase level between samples kept at -70°C to which the reducing agent was added prior to freezing (treatment 4) and those to which the reducing agent was added after thawing (treatment 5), treatment 4 showing a significantly higher level than treatment 5 ($p < 0.001$). The MM isoenzyme activity was significantly greater in samples which were frozen after the addition of dithiothreitol (treatment 4), than in frozen samples to which the reducing agent was added after thawing (treatment 5) and in samples kept at either 4°C or -70°C in the presence of dithiothreitol than in samples kept at the same temperature in the absence of dithiothreitol.

Creatine kinase MB was only observed in samples subjected to treatment 4.

DISCUSSION

In serum, certain substances such as uric acid, oxidised glutathione and albumin act as non-competitive inhibitors of creatine kinase by reversibly oxidising or binding to the active sulphydryl sites in the enzyme. This inhibitory effect protects the enzyme from irreversible inactivation and is itself reversible by the addition of thiol compounds such as dithiothreitol which deblock the active sulphydryl sites of creatine kinase by binding to the inhibitors. In homogenates and isolated mixtures of creatine kinase isoenzymes, the protective effect of the non-competitive inhibitors is not available and inactivation which is not reversible by the use of thiol compounds will occur (Rao, Evans and Mueller, 1977).

My results tend to confirm the views of Rao, Evans and Mueller (1977). In serum samples, as expected, the greatest and only significant difference in total creatine kinase level was observed between samples analysed in the fresh state, after the addition of dithiothreitol and samples subjected to freezing at -70°C in the absence of dithiothreitol, the former having the greater activity. Those two treatments represented the mildest and most severe forms of treatment

respectively. The MM isoenzyme in serum was not affected by sample treatment, which would tend to substantiate the view that it is the most stable creatine kinase isoenzyme (Szasz, Gerhardt and Gruber, 1978). In the case of the MB isoenzyme in serum, fresh samples without dithiothreitol showed significantly lower activity than samples subjected to all other forms of storage. Thus the MB level in fresh samples without dithiothreitol was, as expected, lower than in samples containing the reducing agent.

It is important to note that in serum (a) the presence of dithiothreitol in fresh and frozen samples produced a significant reactivation of the MB isoenzyme and (b) it makes no difference whether dithiothreitol is added before or after storage.

In heart homogenates, all the MB activity was rapidly lost unless the samples were frozen immediately after preparation, in the presence of reducing agent. Samples stored at -70°C without the addition of the reducing agent, could not be reactivated by the addition of dithiothreitol after thawing. The irreversible loss of MB activity was consistent with the proposals by Rao, Evans and Mueller (1977) that the protective effect of certain non-competitive creatine kinase inhibitors, which occur naturally in serum, is not present in isolated mixtures of creatine kinase isoenzymes.

CONCLUSIONS FROM PART 1

It was concluded that in serum, the addition of dithiothreitol was necessary for the reactivation of the MB isoenzyme and that the reducing agent could be added either before freezing at -70°C or after thawing. The inclusion of dithiothreitol before freezing was considered to be preferable.

In tissue homogenates, in order to avoid irreversible inactivation of the MB isoenzyme, dithiothreitol must be added before freezing at -70°C and homogenates should be prepared as quickly as possible to avoid the irreversible inactivation of MB at 4°C which occurs even in the presence of the reducing agent.

PART 2 - TISSUE ISOENZYME LEVELS

INTRODUCTION

From the review of the literature on the normal isoenzyme levels in man and the domestic animals, (see Chapter 5) it became apparent that the absolute lactate dehydrogenase isoenzyme levels in ovine tissues had not been reported, although the percentage distribution was well documented, and that there were no reports of either the percentage distribution or the absolute isoenzyme levels of aldolase, glucosephosphate isomerase or creatine kinase in this species. The aim of this part of the work was two-fold:

(1) to establish the normal absolute values and percentage distribution of the isoenzymes of lactate dehydrogenase, glucosephosphate isomerase, aldolase and creatine kinase in ovine tissues, and

(2) to determine which enzymes, studied either singly or in combination, produced a distinct isoenzyme profile for each tissue.

MATERIALS AND METHODS

Eight, clinically normal, 5½ month old Scottish Blackface lambs were used.

For preparation of haemolysates, venous blood samples were collected using 20g x 1½" Vacutainer

needles and 7ml Vacutainer tubes (Becton-Dickinson Ltd.) containing 143 USP units of lithium heparin. Blood samples were collected immediately before death.

Lambs were then killed by the intravenous injection of 20ml of 20% Pentobarbitone Sodium BP(Vet.) (Euthatal, (R) May and Baker Ltd., Dagenham, Essex) and bled out. Approximately 5g of the following organs were removed from each lamb within 2 hours of death:

(a) liver - dorsal border

(b) heart - left ventricular wall

(c) kidney - full thickness, containing cortex, medulla and renal crest

(d) lung - diaphragmatic lobe

(e) skeletal muscle - long head of triceps brachii

(f) abomasum - full thickness of fundic region

(g) small intestine - full thickness

(h) large intestine - full thickness of elliptical coils

The samples were kept on ice until homogenates were prepared, on the day of collection.

The unclotted blood samples were centrifuged at 3000 rpm for 5 minutes and the plasma and buffy coat removed. The red cells were washed by adding 4 volumes Ringer's Injection (Viaflex, (R)

Travenol Laboratories Ltd., Thetford, Norfolk) to 1 volume of red blood cell pellet, mixing gently by inversion and centrifuging in a high speed refridgerated centrifuge at 14,000 rpm for 10 minutes at 4°C. The supernatant was discarded and 4 volumes distilled water added. The red cell pellet was agitated in distilled water and centrifuged at 14,000 rpm for a further 10 minutes to remove the red cell ghosts.

Fat and connective tissue were removed from tissue samples before homogenisation. After careful rinsing in 0.25M sucrose solution, the mucosa was gently scraped from the abomasal and intestinal samples with the blunt edge of a scalpel blade and the remaining tissue discarded. 2g samples of each tissue were homogenised in 8ml, 0.25M sucrose solution by means of a Griffiths' tube and centrifuged at 14,000 rpm for 30 minutes at 4°C in the high speed centrifuge. The tissue and lysed red cell supernatants were each divided into five 100µl aliquots and one 5ml aliquot. 2µl of 1M dithiothreitol was added to two of the 100µl aliquots before freezing, these samples being used for total creatine kinase and creatine kinase isoenzyme estimations. The remaining three 100µl aliquots were used for lactate dehydrogenase, glucosephosphate isomerase and aldolase isoenzyme studies and the 5ml

aliquot for estimation of the total levels of these three enzymes, and total protein or haemoglobin measurements. Homogenates and haemolysates were stored at -70°C until analysed.

Total enzyme levels were determined after appropriate dilution of samples to ensure linearity of the reaction rate, by the methods previously described. Quantitative isoenzyme estimations were undertaken using the electrophoretic techniques described in Chapter 4.

Total protein or haemoglobin determinations were carried out on homogenates and haemolysates respectively, to allow the expression of total enzyme and isoenzyme levels as IU/g protein or IU/g haemoglobin.

Total protein was determined in undiluted tissue homogenates by the biuret method of Henry, Sobel and Berkman (1957). The protein standard used was Seronorm, batch no. 144 (Nyegaard and Co., Oslo, Norway. U.K. agents, BDH Chemicals Ltd., Poole, Dorset). A blank and a standard were used for each batch of homogenates. The test was conducted as follows:

tube	3% NaOH	sample	biuret reagent
blank	5ml	-	1ml
standard	4.9ml	0.1ml	1ml
unknown	4.9ml	0.1ml	1ml

The tubes were mixed and incubated at room temperature for 15 minutes. The absorbances of the protein-biuret complexes were read against the blank at 545nm in a Cecil CE 292 Digital Ultraviolet Spectrophotometer (Cecil Instruments Ltd.) and the protein concentrations determined as follows:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{protein concentration of standard} = \text{g/l protein}$$

Haemoglobin was determined using a Haemoglobinometer (Coulter Electronics Ltd., Coldharbour Lane, Harpenden, Hertfordshire) and the results expressed in g/l.

RESULTS

Lactate dehydrogenase

TABLE 6.8

LACTATE DEHYDROGENASE ISOENZYMES IN TISSUES - ABSOLUTE LEVELS n = 8

Tissue	TOTAL LDH		LDH_1		LDH_2		LDH_3		LDH_4		LDH_5	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver[1]	177.9	48.76	153.1	37.57	10.8	7.74	10.9	6.93	2.1	2.32	1.1	3.01
heart[1]	2744.8	577.47	2447.8	599.66	160.4	71.67	77.9	81.42	58.2	164.69	0.0	-
kidney[1]	761.6	151.59	615.5	105.52	57.5	17.02	67.1	28.09	14.0	11.54	7.4	10.18
lung[1]	136.0	35.31	42.4	13.90	11.7	5.31	47.5	7.59	16.8	8.28	17.5	21.59
skeletal muscle[1]	4400.4	1403.69	398.8	364.94	160.2	87.65	429.5	286.24	220.0	129.52	3190.8	1425.59
abomasum[1]	977.4	143.03	805.0	171.63	55.0	28.12	92.8	26.25	15.1	10.58	9.8	11.08
small intestine[1]	380.8	41.74	30.6	8.84	25.4	10.13	141.5	39.20	63.2	22.57	120.3	53.99
large intestine[1]	437.0	76.28	100.4	28.24	79.3	35.21	182.9	35.26	45.0	18.93	29.2	8.25
red blood cells[2]	3.8	4.32	3.1	3.40	0.3	0.36	0.5	0.68	0.0	-	0.0	-

For statistical definitions, see page vi.

1 IU/g protein

2 IU/g Hb.

TABLE 6.9.

LACTATE DEHYDROGENASE ISOENZYMES IN TISSUES - PERCENTAGE LEVELS n = 8

Tissue	% LDH_1		% LDH_2		% LDH_3		% LDH_4		% LDH_5	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver	86.4	3.37	5.8	2.92	5.8	2.64	1.1	1.15	0.7	2.12
heart	89.0	7.43	6.4	3.51	2.9	2.81	1.7	4.81	0.0	-
kidney	81.3	4.75	7.5	1.38	8.5	2.52	1.8	1.34	0.8	1.14
lung	31.4	6.96	8.6	3.19	36.1	6.00	12.3	3.56	11.6	12.52
skeletal muscle	10.8	10.75	3.8	2.27	9.7	6.54	5.0	3.25	70.7	16.90
abomasum	81.7	7.61	5.9	3.72	9.7	3.04	1.6	1.11	1.1	1.31
small intestine	8.1	2.33	6.5	2.34	37.2	8.54	16.6	5.68	31.6	12.97
large intestine	23.1	5.52	17.7	4.85	42.0	5.82	10.3	4.07	6.8	2.09
red blood cells	75.4	13.82	6.5	2.94	18.0	11.37	0.0	-	0.1	0.32

For statistical definitions see page vi.

The mean values and standard deviations were calculated for total lactate dehydrogenase and the absolute and percentage isoenzyme levels in each tissue. The results are shown in Tables 6.8 and 6.9. In order to determine whether tissues could be distinguished on the basis of their isoenzyme pattern, paired t-tests were carried out to compare the percentage of each isoenzyme in each tissue with the percentage in each of the other tissues. Paired t-tests were used in preference to analysis of variance to avoid bias as a result of treating values from different tissues as if they were derived from different individuals. The t values and levels of significance are given in Appendix 3.
The statistical comparison of the isoenzyme pattern of each tissue with all other tissues revealed that all tissues except kidney and abomasal mucosa showed a distinct isoenzyme pattern, i.e. kidney and abomasum were indistinguishable on the basis of their lactate dehydrogenase isoenzyme pattern.

Typical tissue lactate dehydrogenase zymograms are shown in Fig. 4.34.

Glucosephosphate isomerase

TABLE 6.10.

GLUCOSEPHOSPHATE ISOMERASE ISOENZYMES IN TISSUES - ABSOLUTE LEVELS n = 8

Tissue	TOTAL GPI		GPI I		GPI II		GPI III	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver[1]	1026.6	149.39	104.9	55.16	200.8	64.01	720.9	144.50
heart[1]	3484.1	1048.39	176.9	75.09	824.1	338.16	2483.4	821.60
kidney[1]	1048.6	167.16	82.4	23.46	327.2	107.92	638.9	134.46
lung[1]	410.2	58.15	33.9	20.74	111.3	31.37	265.0	49.30
skeletal muscle[1]	4398.3	1064.22	345.6	223.15	1143.0	488.73	2909.5	878.43
abomasum[1]	833.8	202.36	94.5	42.86	188.7	56.30	550.7	151.29
small intestine[1]	916.5	89.00	60.9	30.66	205.1	36.25	650.7	102.69
large intestine[1]	690.1	116.07	44.3	29.39	149.6	24.87	496.4	114.37
red blood cells[2]	34.5	12.37	2.5	1.58	6.5	4.19	25.4	7.77

For statistical definitions, see page vi.

1 IU/g protein

2 IU/g Hb

TABLE 6.11

GLUCOSEPHOSPHATE ISOMERASE ISOENZYMES IN TISSUES - PERCENTAGE LEVELS

n = 8

Tissue	% GPI I		% GPI II		% GPI III	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver	10.6	5.76	19.4	4.45	70.1	7.71
heart	5.3	2.50	23.6	7.86	71.2	9.59
kidney	8.1	2.59	30.8	8.68	61.1	9.10
lung	8.1	3.76	27.3	7.98	64.6	9.37
skeletal muscle	7.8	3.66	26.2	8.74	66.0	10.25
abomasum	11.7	5.73	22.9	5.16	65.3	4.52
small intestine	6.7	3.50	22.5	4.27	70.8	6.99
large intestine	6.7	4.68	21.8	2.93	71.5	5.74
red blood cells	7.2	4.22	17.9	5.54	74.9	6.89

For statistical definitions, see page vi.

Three glucosephosphate isomerase isoenzymes were present in ovine tissues. The bands were numbered GPI I, GPI II and GPI III, the former having the greatest anodic mobility.

The mean values and standard deviations of total glucosephosphate isomerase and the absolute and percentage levels of its isoenzymes were calculated, the results being presented in Tables 6.10 and 6.11. As described for lactate dehydrogenase, paired t-tests were performed to compare the percentage levels of glucosephosphate isomerase isoenzymes between tissues.

The t values and levels of significance are given in Appendix 3.

Only red blood cells showed a unique glucosephosphate isomerase isoenzyme pattern with a small secondary peak immediately anodal to, and overlapping with GPI II. In order to allow comparison between the percentage of GPI II in red blood cells and that of other tissues, the activity in the secondary peak was included in the value for GPI II. Excluding red blood cells, the results of the paired t-tests indicated that no tissue showed a unique isoenzyme pattern. Fig. 4.37 shows typical zymograms of tissue glucosephosphate isomerase isoenzymes.

Aldolase

The mean and standard deviation of the total aldolase activity in each tissue is given in Table 6.12.

Aldolase showed diffuse, overlapping areas of activity for each tissue on electrophoresis. The mean anodal migration distances from the origin were calculated for the cathodal and anodal edges of each area of aldolase activity in each tissue and the results are shown in Table 6.13, negative values indicating cathodal migration. The number of individuals showing each arbitrary zone is given in square brackets after each mean value. A numbering system was adopted for the zones, zone 1 being the most anodal. In any

one tissue, the number of areas of activity present varied between individuals and in some cases, several zones overlapped to such an extent that they were indistinguishable.

TABLE 6.12

TOTAL ALDOLASE ACTIVITY IN TISSUES n = 8

Tissue	TOTAL ALDOLASE	
	$\bar{x}$	s
liver[1]	21.1	3.48
heart[1]	38.5	14.18
kidney[1]	21.8	5.56
lung[1]	2.4	1.77
skeletal muscle[1]	616.6	271.02
abomasum[1]	16.6	15.34
small intestine[1]	18.5	17.18
large intestine[1]	39.7	15.21
red blood cells[2]	1.1	0.47

For statistical definitions, see page vi.

1 - IU/g protein

2 - IU/g Hb

TABLE 6.13.

MIGRATION DISTANCE OF ZONES OF ALDOLASE ACTIVITY n = 8

Tissue	mean migration distance (mm) from origin zone 1	zone 2 a b c	zone 3	zone 4	zone 5
liver		31 -43 [6]	0-27[8]		
heart	45-55[4]	32-44[5]	19-32[4]	9-19[4]	-3-9[6]
kidney	42-52[3]		3-31[8]		-5-3[8]
lung	44-56[4]	27-44[4]	13-31[6]	3-13[7]	-3-3[8]
skeletal muscle	50-60[6]	35-47[3]	13-33[4]	-4-11[8]	
abomasum	47-59[6]	13-45[5]		2-14[4]	-3-3[6]
small intestine	42-53[6]		4-35[7]		-4-3[8]
large intestine	49-58[6]	25-45[7]	1-25[5]		-4-1[6]
red blood cells	24-55[8]		←	-2-24[8]	→

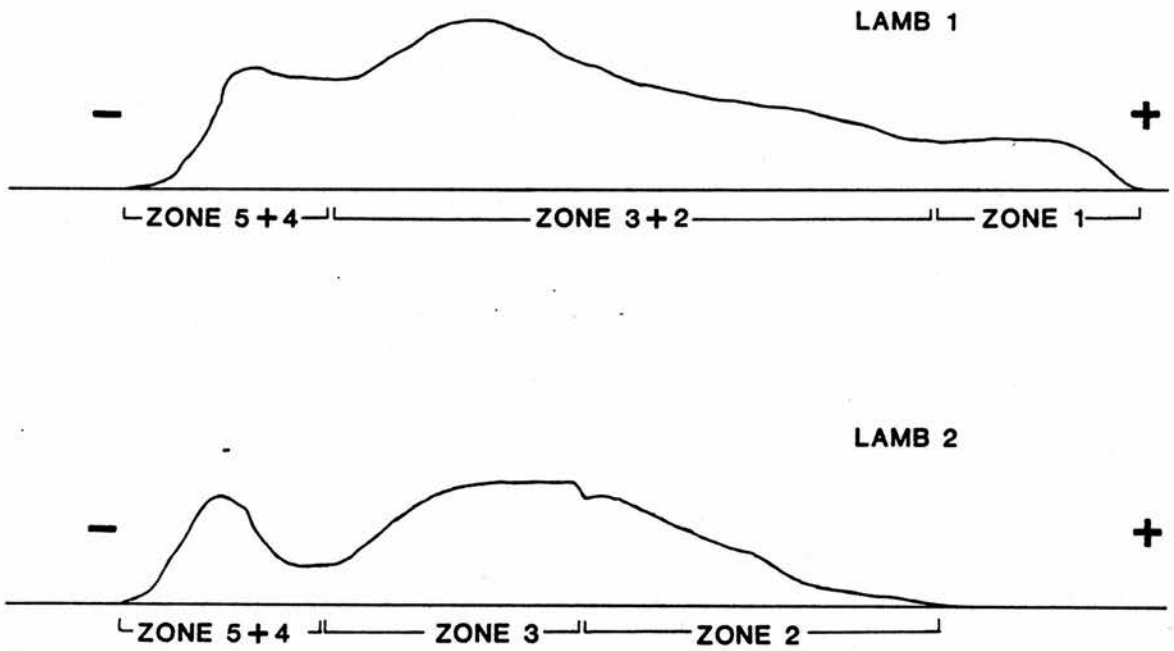
a - mean distance of cathodal edge of zone from the origin

b - mean " " anodal " " " " " "

c - number of individuals showing the zone

Figure 6.1. Fluorometric scans of skeletal muscle aldolase from two lambs showing discrepancies in the number of zones of activity present and demonstrating overlapping between adjacent zones.

SKELETAL MUSCLE ALDOLASE SCANS



Liver showed 1-2 zones, heart 2-5, kidney 2-3, lung 3-5, skeletal muscle 2-4, abomasum 2-4, small intestine 2-3, large intestine 2-4 and red blood cells 2 zones of activity. Fig. 4.36 shows typical zymograms of aldolase in sheep tissues.

The means and standard deviations of the absolute activity and percentage values in the zones, were not calculated in view of the fact that in many cases, zones overlapped and it was impossible to quantify them individually. For this reason, and because zones observed in different tissues did not correspond, paired t-tests to compare the percentage values of each zone in different tissues could not be carried out. Examples of fluorometric scans of aldolase in tissues are shown in Fig. 6.1.

Creatine kinase

Up to five bands of creatine kinase activity were visible after electrophoresis of ovine tissues which were named BB, MB_1, MB_2, MM_1 and MM_2. The latter migrated cathodally from the origin whereas the other four bands migrated anodally, BB showing the greatest anodic mobility.

The mean values and standard deviations of the total enzyme and isoenzyme levels were calculated for each tissue and the results are shown in Tables 6.14 and 6.15. Again, paired t-tests were carried out to compare the percentage values of creatine kinase

TABLE 6.14.

CREATINE KINASE ISOENZYMES IN TISSUES - ABSOLUTE VALUES n = 8.

Tissue	TOTAL CK		MM_2		MM_1		MB_2		MB_1		BB	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver[1]	77.4	21.54	13.0	4.06	22.4	16.63	3.2	0.53	1.3	1.34	37.5	13.08
heart[1]	20429.9	5295.16	0.0	-	20429.9	5295.16	0.0	-	0.0	-	0.0	-
kidney[1]	188.0	54.53	0.0	-	19.1	14.78	68.4	19.98	26.5	9.99	73.9	33.85
lung[1]	194.1	59.57	2.1	6.08	26.0	9.12	0.0	-	28.3	18.48	137.6	53.98
skeletal muscle[1]	39746.7	15942.36	0.0	-	39746.7	15942.36	0.0	-	0.0	-	0.0	-
abomasum[1]	2959.2	1474.62	0.0	-	116.1	61.50	0.0	-	0.0	-	2843.1	1431.38
small intestine[1]	1737.87	507.89	7.7	16.26	51.7	36.79	0.0	-	211.5	183.46	1466.4	478.71
large intestine[1]	1382.6	338.05	0.0	-	184.1	100.52	0.0	-	139.9	67.65	1058.7	312.67
red blood cells[2]	27.6	6.28	0.0	-	4.1	3.82	0.0	-	19.7	5.67	3.9	2.00

For statistical definitions, see page vi.

1 IU/g protein

2 IU/g Hb

TABLE 6.15.

CREATINE KINASE ISOENZYMES IN TISSUES - PERCENTAGE LEVELS n = 8

Tissue	%MM_2		%MM_1		%MB_2		%MB_1		%BB	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
liver	16.9	2.57	27.5	12.69	4.3	1.28	1.8	1.43	49.4	12.71
heart	0.0	-	100.0	-	0.0	-	0.0	-	0.0	-
kidney	0.0	-	9.7	5.28	37.9	10.17	14.0	3.46	38.3	8.04
lung	1.4	4.07	14.7	7.15	0.0	-	14.5	6.34	69.3	9.34
skeletal muscle	0.0	-	100.0	-	0.0	-	0.0	-	0.0	-
abomasum	0.0	-	5.5	5.87	0.0	-	0.0	-	94.5	5.87
small intestine	0.5	1.08	3.5	2.76	0.0	-	11.7	8.99	84.2	10.19
large intestine	0.0	-	14.4	8.79	0.0	-	9.8	3.33	75.8	8.78
red blood cells	0.0	-	13.7	10.45	0.0	-	71.6	12.04	14.7	7.94

For statistical definitions, see page vi.

isoenzymes between tissues, the t values and levels of significance being given in Appendix 3.

Mean total creatine kinase was highest in skeletal muscle, followed by heart muscle, abomasum, small intestine, large intestine, lung, kidney, liver and red blood cells. From Table 6.15, the isoenzyme distribution varied markedly between tissues and the results of the paired t-tests indicated that only heart was indistinguishable from skeletal muscle and lung from large intestine.

The identity of the five bands present in ovine tissues was investigated further by comparing the migration distances of the bands with those of CPK Isotrol$^{(R)}$ (Sigma Chemical Co.), an electrophoretic marker for the identification of human serum and tissue MM, MB and BB isoenzymes. The MM isoenzyme of the electrophoretic marker corresponded to MM_1 in ovine tissues, the MB isoenzyme to MB_2 while the BB isoenzyme corresponded to the BB isoenzyme of ovine origin.

Since the possibility existed that the unexpected appearance of five rather than three isoenzymes in ovine tissues might be artefacts due to the addition of dithiothreitol to prevent inactivation during storage, fresh homogenates of samples of ovine liver and kidney were subjected to electrophoresis within 10 minutes of the animal's slaughter, without the

addition of the reducing agent. Again, all five bands were present in liver and MM_1, MB_2, MB_1, and BB in kidney.

Typical creatine kinase zymograms in ovine tissues are shown in Fig. 4.41.

DISCUSSION

The activity of lactate dehydrogenase in IU/g protein has not been previously reported in ovine tissues although the relative activity of this enzyme has been discussed by Keller (1973) who found that myocardium showed the greatest activity, followed by skeletal muscle, kidney cortex, brain and liver, and by Márquez et al. (1977) who found skeletal muscle to have the highest level, followed by myocardium, kidney and liver. Keller measured the levels in superficial gluteus muscle whereas Márquez et al. did not state the muscle from which samples were obtained and this may account for the difference between these authors in the tissue with the highest lactate dehydrogenase activity as the total activity is known to vary markedly in different skeletal muscles (Briand et al., 1981). My observations agree with those of Márquez et al. (1977) for the limited number of tissues which they studied, the tissues in descending order of lactate dehydrogenase activity

being skeletal muscle, heart muscle, abomasal mucosa, kidney, large intestine mucosa, small intestine mucosa, liver, lung and red blood cells. The relatively high activity in the digestive tract, particularly the abomasum, was of interest in view of the absence of reports on the measurement of this enzyme in the serum in gastrointestinal tract disease in the sheep.

As previously mentioned, the absolute levels of lactate dehydrogenase isoenzymes in ovine tissues do not appear to have been previously reported. The percentage distribution in a limited number of tissues has however, been described (Boyd, 1964 ; Paulson, Pope and Baumann, 1966; Tollersrud, 1970; Briand et al., 1981). The percentage values obtained by these authors in liver, heart, lung and triceps brachii muscle (see Appendix 2, Table 5.8) are in close agreement with the present findings with LDH_1 being present in the greatest proportion and in general, consecutively lower proportions of LDH_2, LDH_3, LDH_4 and LDH_5 in liver, heart and kidney, approximately equal proportions of LDH_1 and LDH_3 and lower levels of LDH_2, LDH_4 and LDH_5 in lung, and a predominance of LDH_5 followed by LDH_1 or LDH_3 and LDH_2 or LDH_4 in triceps brachii.

The lactate dehydrogenase isoenzyme distribution in the abomasum, small intestine, large intestine and red blood cells has apparently not been determined in ruminants. In the pig, the fundic mucosa of the

stomach contains approximately equal amounts of LDH_1 and LDH_3 (35.7 and 30.9%, respectively) with lower proportions of LDH_2, LDH_4 and LDH_5 (Timms and Prochazka-Perthen, 1975), but this pattern bears little resemblance to that of the fundic mucosa of the ovine abomasum which showed a marked predominance of LDH_1.

In the small intestine mucosa of dogs and man, all five isoenzymes are present in approximately equal proportions (Graeber et al., 1981), whereas in the horse, the more cathodic isoenzymes (LDH_3, LDH_4 and LDH_5) predominate (Thornton and Lohni, 1979). In the sheep the distribution is similar to that of the horse and may reflect a lower energy requirement from oxidative phosphorylation (favoured by the LDH isoenzymes containing mainly H subunits) in the small intestine of herbivores since active transport of glucose across the intestinal mucosa is minimal in herbivores, most of their glucose being formed by gluconeogenesis in the liver and kidney.

The large intestine mucosa of canine, human and equine origin shows a similar lactate dehydrogenase isoenzyme pattern to the small intestine in these species (Littlejohn and Blackmore, 1978; Graeber et al., 1981), but in the sheep, the distribution in large intestine was found to be unlike that of other species, with the anodic isoenzymes, LDH_1, LDH_2 and LDH_3, being present in the highest concentration, particularly the latter, which constituted 42% of the total enzyme

activity.

Human and equine red blood cells show a marked predominance of LDH_1 and LDH_2, with little or no activity of the other three isoenzymes (Wieme and Van Maercke, 1961; Vesell, 1961; Coffman, Mussman and Cawley, 1968; Anderson, 1976; Thornton and Lohni, 1979). The present findings indicate that although LDH_1 constitutes 75% of the lactate dehydrogenase activity in sheep red cells, most of the remainder is LDH_3 and clearly increases in the proportion of these two isoenzymes may be expected in serum samples containing haemolysed red blood cells.

Total glucosephosphate isomerase has not been quantified in the tissues of sheep except in haemolysates, Smith and Lee (1974) reporting a value of 17.8 ± 2.74 IU/g Hb at 25°C (approximately 22.2 IU/g Hb at 30°C) in sheep red cells. In the liver of ruminating calves, Pearce and Unsworth (1980) found the total enzyme level to be 573-599 IU/g protein at 37°C (approximately 401-419 IU/g protein at 30°C), while in human heart the normal value is said to be 1280 IU/g protein at 30°C (Gracy and Tilley, 1975).

In this experiment, skeletal muscle showed the greatest total glucosephosphate isomerase level followed by heart muscle, kidney, liver, small intestine mucosa, abomasal mucosa, large intestine mucosa, lung and red blood cells. The values obtained in ovine liver and

heart were higher than those reported in calves and man respectively, and the level in red blood cells (34.5 ± 12.37 IU/g Hb) was higher than the value previously reported in the sheep. The latter may be attributable to differences in the allelozymes of glucosephosphate isomerase in the sheep used in the present experiment and those used by Smith and Lee (1974) since, at least in rabbits and man, genetic variants of this enzyme show different total enzyme activities in the red blood cells (Welch, Fitch and Parr, 1970; Harris and Hopkinson, 1976).

Three bands of glucosephosphate isomerase activity have been noted on electrophoresis of ovine haemolysates (Carter and Detter, cited by Yoshida and Carter, 1969) and one band in liver and kidney (Baker and Manwell, 1977) but this is the first report of their quantitation in ovine tissues. The isoenzymes, which I have named GPI I , II and III, were present in essentially the same proportions in all tissues examined, with the exception of red blood cells which were unique in showing a small secondary band overlapping with GPI II. The reason for the presence of this band is not known but is not due to interference from the haemoglobin zone.

Total aldolase levels do not seem to have been measured in ovine tissues although Gerber (1965) and Anderson (1976) have measured aldolase activity

(IU/g wet weight of tissue at 25°C) in equine tissues. The results obtained by Gerber for the tissues which I examined in sheep were, in descending order of activity, skeletal muscle, liver, kidney, large intestine, small intestine and heart, whereas Anderson found the order to be skeletal muscle, red blood cells, kidney, liver, and heart. The present findings in ovine tissues differ from both these reports with skeletal muscle showing by far the greatest activity, followed by large intestine mucosa, heart, kidney, liver, small intestine mucosa, abomasal mucosa, lung and red blood cells.

Qualitative aldolase isoenzyme studies in human tissues have produced unequivocal results, for example Pietruszko and Baron (1967) showed that liver aldolase consisted of two bands, and skeletal muscle and spleen aldolase of one band corresponding to the most anodally migrating of the two liver bands, whereas kidney showed two sub-bands in the same position as the single band of muscle and spleen. Lebherz and Rutter (1969) reported only aldolase A in muscle and spleen, the five isoenzymes of the A-C hybrid set in heart and brain, aldolase A and B in liver and the five isoenzymes of the A-B hybrid set in kidney.

Sheedy and Masters (1969), using starch gel electrophoresis, reported a unique complement of aldolase isoenzymes in bovine and ovine tissues compared with that of other species, with all five members of

the A-C hybrid set being present in liver, kidney, skeletal muscle, heart, lung, spleen and brain, with kidney showing in addition, the A-B hybrid set giving a total of nine isoenzymes and with liver also showing aldolase B.

In the present study, aldolase in ovine tissues consisted of diffuse, overlapping areas of aldolase activity on polyacrylamide gel electrophoresis. Up to five areas were present in heart and lung, four in skeletal muscle, abomasal mucosa and large intestine mucosa, three in kidney and small intestine mucosa, and two in liver and red blood cells. In Table 6.13, the diffuse bands shown by each tissue have been categorised into zones according to their mean migration distance from the origin, but all tissues except heart and lung showed overlapping of zones so that they were indistinguishable on the fluorometric tracing, making quantitation impossible.

The migration distance and degree of separation of aldolase isoenzymes, like all proteins, depends on the electrophoresis medium and on the buffer system employed. The buffer system used in the present experiment was that of Harris and Hopkinson (1976) who found that aldolase C migrated the furthest anodally using this system, albeit in starch gel, followed by the A-C hybrids, with aldolase A showing the least anodal migration. The position of aldolase

B was intermediate between aldolase A and the hybrid A_3C. The absence of the least anodally migrating zone (zone 5) in liver samples in the present experiment suggests that this zone represents aldolase A which is present in most tissues but is not detectable or is present at only a very low concentration in liver. Aldolase C, which migrates the furthest anodally (Harris and Hopkinson, 1976) is likely to be represented by zone 1. The exact identity of zones 2, 3 and 4 is less certain, but their position strongly indicates that they correspond to the A-C hybrid isoenzymes and aldolase B. Thus, my findings are consistent with the report by Sheedy and Masters (1969) describing the presence of the A-C hybrid set in all ovine tissues which he studied. However, in view of the close proximity of the expected positions of aldolase A and B using the technique employed in the present investigation, the detection of the A-B hybrid set which these authors described in ovine kidney would not be possible.

The degree of separation of aldolase isoenzymes and the resolution obtainable on electrophoresis varies markedly between authors. Penhoet et al. (1967), for example, found sharply resolved bands on cellulose acetate electrophoresis of purified isoenzymes and more diffuse but better separated bands in 3.5% acrylamide gels. In starch gel, Pietruszko

and Baron (1967) and Schapira and Nordmann (1969) found diffuse bands which in the former appeared to be overlapping. Polyacrylamide gel was chosen for the present study as it is said to give superior resolution to other media (Dietz and Lubrano, 1967; Gordon, 1969), but there are few previous reports of its use for aldolase. The results obtained in this experiment indicated that polyacrylamide gel is apparently not the medium of choice for aldolase isoenzyme separations but attempts to improve the results by the use of other media such as cellulose acetate were not made since (a) there was insufficient time available and (b) all tissues were distinguishable on the basis of their lactate dehydrogenase and creatine kinase isoenzyme distribution.

Total creatine kinase levels in ovine tissues have been estimated by Brown and Wagner (1968) in Sigma units/100mg wet weight of tissue and by Boyd (1976) in IU/g wet weight of tissue at 25°C. Although the results obtained by these authors cannot be compared directly with the results of this experiment, Brown and Wagner found that skeletal muscle showed the highest activity, followed by duodenum, myocardium, abomasum, kidney, lung, distal colon and liver (of the tissues which I examined) whereas Boyd found the decreasing order of activity to be skeletal muscle,

heart, small intestine, lung, kidney and liver. In the present study, skeletal muscle showed the highest activity followed by heart, abomasal mucosa, small intestine mucosa, large intestine mucosa, lung, kidney, liver and red blood cells. My findings were similar to those of Boyd (1976) with muscular tissue showing the greatest creatine kinase activity, digestive and respiratory tracts showing intermediate levels and the parenchymatous organs having the least activity. The relatively high activity in the digestive tract was noted since even in human medicine, this enzyme is rarely studied in diseases affecting these organs, although the human small and large intestine also show considerable creatine kinase activity (Graeber et al., 1981).

The absolute or percentage levels of the isoenzymes of creatine kinase have not been previously reported in ovine tissues. The isoenzyme distribution in man has been described by Tsung (1976), Ogunro, Hearse and Shillingford (1977) and Graeber et al. (1981) (for details, see Chapter 5) and in horses by Argiroudis, Kent and Blackmore (1982), but the results obtained differ quite markedly between human, equine and ovine tissues, and only the present report describes the existence of five distinct bands. The existence of a separate mitochondrial isoenzyme in addition to the three

cytoplasmic isoenzymes was recognised by Jacobs, Heldt and Klingenberg (1964) in various rat tissues, while Somer et al. (1974) reported that mitochondrial creatine kinase from rat heart contained MM and a second isoenzyme which migrated just cathodally to the MM band. Sanders, Joung and Rochman (1976) also noted this isoenzyme in rat and human heart homogenates, and a band which migrated cathodally but to a lesser extent than the cathodic heart band, in kidney and brain extracts. The authors considered the cathodic isoenzyme in heart could have originated from cell organelles, but did not suggest a source of the cathodic band in kidney and brain.

Heterogeneity of the anodally migrating isoenzymes of creatine kinase have been described in rabbit skeletal muscle by Cattan et al. (1978) who observed three equally spaced bands on isoelectric focusing which were thought to be due to the existence of two different types of M subunit, giving rise to two homodimers and one heterodimer. Two types of M and B subunit have also been reported in chicken and mouse tissues (Rosenberg, Eppenberger and Perriard, 1981). Chapelle and Heusghem (1980) reported the presence of two MM isoenzymes, MM and MM_1 in human myocardium and skeletal muscle subjected to isoelectric focusing, MM_1 migrating anodally to MM. Similarly, Yasmineh, Yamada and Cohn (1981) observed

three MM isoenzymes on isoelectric focusing of human heart extracts. Hybridisation of the mitochondrial isoenzyme with one of the cytoplasmic isoenzymes might be responsible for the appearance of additional bands, for example, Wevers, Mul-Steinbush and Soons (1980) observed that human heart mitochondrial isoenzyme hybridised with BB to form an isoenzyme with a mobility slightly less anodic than MB.

In the present investigation, the identity of the five bands observed was investigated in relation to three criteria. Firstly, the staining procedure was known to be specific for creatine kinase, therefore all five bands represented creatine kinase activity and were not due to interference from adenylate kinase. Secondly, all five bands found in liver and the four bands in kidney were present in samples prepared in the absence of dithiothreitol, which were not subjected to freezing at -70°C and were separated by electrophoresis within 10 minutes of slaughter of the sheep. Thirdly, the migration distances of the bands were compared with those of a human creatine kinase electrophoretic marker.
The position of MM_2 indicates that it is likely to represent the mitochondrial isoenzyme, but the identity of the two isoenzymes which were named MB_1 and MB_2 is less certain. The fact that MB_1

occurred more consistently in the tissues than MB_2 tends to indicate that it corresponds to the human MB isoenzyme, although differing in electrophoretic mobility, while MB_2 which was found only in kidney and liver may be regarded as the "extra" MB band. The identification of the type of subunits of which the two "MB" isoenzymes are composed by immunological means would be required to determine the nature of these two bands, but such procedures were outwith the scope of this study.

CONCLUSIONS

The absolute levels of lactate dehydrogenase and the absolute and percentage levels of glucose-phosphate isomerase and creatine kinase isoenzymes in ovine tissues have been reported for the first time. Five lactate dehydrogenase isoenzymes were present, as in other species, three glucosephosphate isomerase isoenzymes, five diffuse zones of aldolase activity and five creatine kinase isoenzymes. The cathodally migrating creatine kinase isoenzyme was considered to be of mitochondrial origin.

Statistical analysis revealed that only red blood cells showed a unique glucosephosphate isomerase pattern, whereas only kidney and abomasum showed indistinguishable lactate dehydrogenase patterns. Creatine kinase isoenzyme patterns allowed distinction

between all tissues except heart and skeletal muscle, and lung and large intestine. Aldolase was not investigated further since it showed an inconsistent number of zones of activity in each tissue. Lactate dehydrogenase in conjunction with creatine kinase isoenzyme estimations allowed all the tissues studied to be distinguished. Determination of the isoenzyme levels of these two enzymes in the serum in combination, may have potential in the detection of tissue-specific serum isoenzyme profiles following tissue damage in sheep.

PART 3 - THE EFFECTS OF PHYSIOLOGICAL PARAMETERS ON SERUM ISOENZYME LEVELS

INTRODUCTION

Little information has been published on the effect of physiological parameters on serum lactate dehydrogenase levels in normal sheep, while no such information is available for serum glucosephosphate isomerase.

In the present experiment, which was carried out in conjunction with the East of Scotland College of Agriculture, the effect of feeding ewes in the last four weeks of pregnancy on two forms of concentrate differing in undegradable protein content, with silage as a basal diet, was assessed. Two lambing management regimen were superimposed and assessment made of lamb survival and performance. In addition, I sampled nine rams, at least four of which had been mated to the experimental ewes. The project enabled me to investigate the effect of various physiological factors on serum isoenzyme levels, viz.

(a) serum isoenzyme levels in ewes in relation to time after parturition

(b) serum isoenzyme levels in lambs in relation to sex, age and growth rate

(c) a comparison between serum isoenzyme levels in rams, ewes and lambs

(d) an investigation into the possible existence of genetic variants of glucosephosphate isomerase.

EXPERIMENTAL DESIGN

Two groups each of 50 Scottish Halfbred ewes aged 3-6 years and in lamb to Suffolk rams were housed indoors in separate straw yards for the last four weeks of pregnancy. All ewes received silage as the basal diet at 3.5kg/head/day. Group L (low protein) ewes also received a concentrate diet consisting of 97.5% barley and 2.5% minerals at 450g/head/day, while group H (high protein) ewes received 72.5% barley, 10% sugar beet pulp, 15% fishmeal and 2.5% minerals at the same level of feeding. Concentrate composition was as follows:

		in DM			
	DM(g/kg)	ME(MJ/kg)	CP(g/kg)	RDP(g/kg)	UDP(g/kg)
Low protein concentrate	851	12.7	114	86	28
High protein concentrate	864	12.5	216	130	98

DM = dry matter

ME = metabolisable energy

CP = crude protein

RDP = rumen degradable protein

UDP = undegradable protein

At parturition, half of the lambs from each group received 50ml of bovine colostrum by stomach tube while the remaining lambs were not given extra colostrum. All

lambs were allowed to suckle normally. Lamb groups were classified as follows:

NL - no extra colostrum/lambs from low protein concentrate fed ewes

EL - extra colostrum/lambs from low protein concentrate fed ewes

NH - no extra colostrum/lambs from high protein concentrate fed ewes

EH - extra colostrum/lambs from high protein concentrate fed ewes

After parturition, ewes and lambs were housed in individual pens and were put out to pasture after a few days. Lambs were castrated with rubber rings at approximately one day old.

PARAMETERS INVESTIGATED

Lamb birthweight, sex and litter size were recorded immediately after parturition. Lambs were weighed and serum samples collected at 24 - 36 hours after parturition and at 1-3 weeks, 3-5 weeks and 8-11 weeks of age while serum was collected from ewes at 24-36 hours, 3-5 weeks and 8-11 weeks post-partum. These time intervals will hereafter be referred to as birth (or parturition in ewes), 2 weeks, 4 weeks and 10 weeks. The wide age range of lambs at each sampling was unfortunately unavoidable as they were kept at grass in large groups mixed with other sheep and could not be gathered for sampling at more frequent intervals. Serum samples were obtained on one occasion from nine, adult Suffolk rams. Total lactate dehydrogenase and glucose-

phosphate isomerase assays and estimations of their isoenzymes were carried out on serum samples from rams, ewes and lambs. Samples were not collected from any sheep which was clinically abnormal.

MATERIALS AND METHODS

Venous blood samples were collected from rams, ewes and lambs using 21g x 1½" Vacutainer needles into 7ml, plain Vacutainer tubes (Becton-Dickinson Ltd.). Samples were allowed to clot at ambient temperature and centrifuged at 3000rpm for 5 minutes after which the serum was harvested. Two 100µl aliquots of serum were placed in 0.5ml polypropylene microcentrifuge tubes for lactate dehydrogenase and glucosephosphate isomerase isoenzyme studies, while the remaining serum was placed in a 75x 12mm polypropylene tube for total enzyme estimations. Samples were stored at -70°C until analysed.

Total lactate dehydrogenase and glucosephosphate isomerase were estimated using commercially prepared kits (LDH-L-SVR, Calbiochem-Behring; 355-UV, Sigma Chemical Co., respectively). The change in absorbance at 340nm was measured spectrophotometrically in a Cecil CE 292 Digital Ultraviolet Spectrophotometer (Cecil Instruments Ltd.) using 1cm plastic cuvettes. The spectrophotometer was connected to a TE-7 Tempette circulating water bath (MacKay and Lynn Ltd.) set at 30°C. Accuracy of the total enzyme estimations

was assessed by means of the quality control solution, Sigma Enzyme Control 2-N (Sigma Ltd.).

Isoenzymes were separated and quantified as described on pages 130 and 134. Total enzyme and isoenzyme estimations were not carried out on sera with visible haemolysis in view of the activity of lactate dehydrogenase and glucosephosphate isomerase in red blood cells (see Chapter 6, Part 2).

The mean total serum lactate dehydrogenase and glucosephosphate isomerase levels and their isoenzyme levels, expressed both as absolute values (IU/l) and as a percentage of the total activity were calculated for all ewe, ram and lamb groups in Parts A to D. Unless stated otherwise, results from the same animals at different time intervals were analysed by paired t-tests and results from different animals were analysed by Student's t-test. In view of the large amount of statistical data, the results are only shown in tabular form where they demonstrate a point of interest. All tables from Part 3 are presented in Appendix 4.

A. NORMAL ISOENZYME LEVELS IN EWES

Nine ewes from each of the two nutritional groups were sampled at intervals after lambing. Only 15 of the 18 ewes however, were sampled on each occasion. The results from the two nutritional groups were amalgamated because they showed no significant difference in total enzyme or isoenzyme levels.

RESULTS

The mean values and standard deviations of the total enzyme and isoenzyme levels are shown in Tables 6.43 and 6.45. The results of the paired t-tests to compare the results for lactate dehydrogenase at intervals after lambing are given in Table 6.44. For brevity, only the t values and levels of significance are given.

From Table 6.44, total lactate dehydrogenase was higher at 10 weeks post-partum than at earlier samplings, mainly as a result of increasing LDH_1 and LDH_2 levels. When expressed as a percentage, no clearcut trend emerged although percentage LDH_3 was significantly higher at parturition than at subsequent samplings ($p < 0.01$).

All ewes had three glucosephosphate isomerase isoenzymes, GPI III showing the greatest activity, followed by GPI II and GPI I. Paired t-tests revealed no clear trend in absolute or percentage isoenzyme levels. The absolute level of GPI I was lower one day after parturition and GPI III higher at 10 weeks than at the other sampling times, but these changes did not significantly alter the total enzyme level. When expressed as a percentage, glucosephosphate isomerase isoenzyme levels fluctuated with time after parturition.

DISCUSSION

The normal values reported for total serum lactate dehydrogenase in adult sheep vary markedly between authors, most of whom fail to distinguish between the values obtained for ewes and rams. Márquez et al. (1977) reported a value of 64.5 IU/l at 25°C (approximately equivalent to 85.8 IU/l at 30°C in adult sheep, while Boehringer (1979) reported the normal level to be 530 IU/l at 25°C (approximately 705 IU/l at 30°C). My results fall between these two extremes, with mean values of 192.2, 189.7 and 283.3 IU/l at 30°C shortly after parturition and at 4 weeks and 10 weeks post-partum. The reason for the marked discrepancy between the results reported by Márquez et al. (1977), Boehringer (1979) and those obtained in this experiment is not clear but may be associated with a combination of the different assay procedures employed and breed and age differences. Márquez et al. used adult ewes and rams of unstated breed and age, while the results cited by Boehringer were obtained from 3-6 year old German Merino-Landschaf ewes.

The serum lactate dehydrogenase isoenzyme pattern in adult ewes reported by Tollersrud (1970) and Tollersrud, Baustad and Flatlandsmo (1971) indicate that LDH_1 showed the greatest activity, followed by

either LDH_2 or LDH_4, while LDH_5 showed the least activity. The results obtained in the present experiment were in agreement with those of the above authors, for example Tollersrud, Baustad and Flatlandsmo (1971) obtained percentage values of 50.1 (LDH_1), 7.3 (LDH_2), 28.8 (LDH_3), 9.2 (LDH_4) and 4.6 (LDH_5) in non-gravid ewes while my results for ewes at 10 weeks post-partum were 54.3 (LDH_1), 10.2 (LDH_2), 21.1 (LDH_3), 8.5 (LDH_4) and 6.0 (LDH_5).

Little information is available describing changes in total serum lactate dehydrogenase or its isoenzymes in relation to parturition and lactation in any species. Makkonen, Pentillä and Castrén (1980) reported an increase in the percentage of LDH_5 in the serum of women during labour, which they attributed to release from the myometrium. Frahm et al. (1978) found a significant positive correlation between milk yield and percentage of H subunits of lactate dehydrogenase in the bovine liver. They proposed that high milk yield brought about an increase in gluconeogenesis in the liver from fatty acids, with pyruvate as the intermediate product, the subsequent conversion of pyruvate to lactate being favoured by lactate dehydrogenase isoenzymes containing predominantly H subunits.

In sheep, a significant increase in total serum lactate dehydrogenase has been reported within 24

hours of lambing, compared with the one day pre-partum level (Healy and Falk, 1974). Following this initial increase, activity remained constant for at least the first three weeks of lactation, but the levels were not followed beyond three weeks post-partum.

Unfortunately, in my experiment samples were not collected before parturition. This should have been carried out in order to allow a comparison to be made between pre-partum and post-partum total enzyme and iso-enzyme levels. However, it was observed that in the post-parturient ewe a pattern of changes in total and absolute lactate dehydrogenase isoenzyme levels in the serum emerged. In the first 4 weeks of lactation, neither total enzyme nor isoenzyme levels changed significantly compared with the levels in the immediate post-parturient period. This finding was in agreement with the report by Healy and Falk (1974). The highly significant increase in total serum lactate dehydrogenase at 10 weeks in comparison with the level at parturition and 4 weeks post-partum was associated, in particular, with absolute increases in LDH_1 and LDH_2 (Table 6.44). Since LDH_1 and LDH_2 have subunit structures HHHH and HHHM respectively, it would be expected that an increase in the H:M subunit ratio would also be observed. When the H:M subunit ratios were calculated for parturition,

4 weeks and 10 weeks post-partum from Table 6.43 the values obtained were 2:1, 2.5:1 and 2.9:1 respectively. This finding is of interest with respect to the observation by Frahm et al. (1978) that the proportion of H subunits of lactate dehydrogenase in the liver was positively correlated with milk yield in cattle. However, Frahm et al. did not determine whether this increase was also reflected in the H subunit proportion in the serum.

Milk yield in ewes is reported to reach a peak at 2-3 weeks after lambing, followed by a gradual decline, although it is still about 50-60% of the peak level at 10-12 weeks after lambing (Owen, 1976). Thus, the observation that an increasing proportion of H subunits was found in the serum of ewes as lactation proceeded, at least to 10 weeks after lambing, cannot be solely associated with milk yield. Lactation is known to be maintained by the hormone prolactin and to a lesser extent by adrenocorticotrophic hormone (ACTH), growth hormone and thyroxine.

ACTH also stimulates gluconeogenesis by inducing the release of glucocorticoids from the adrenal cortex which reduce protein synthesis and increase the amino acids available to the liver for gluconeogenesis, in association with a concomitant increase in lipolysis in adipose tissue. By this means, ACTH supports lactation by providing a supply of milk

precursors (Bell, Davidson and Emslie-Smith (1972). My observation that the proportion of H subunits of lactate dehydrogenase in the serum of ewes increased as lactation proceeded is consistent with the view that gluconeogenesis is stimulated during lactation (Bell, Davidson and Emslie-Smith, 1972; Gow, McDowell and Annison, 1981) since an increased proportion of H subunits in the liver also favours gluconeogenesis (see Chapter 2). This concept assumes that the changing serum isoenzyme profile reflects that of the tissues.

Total glucosephosphate isomerase and its isoenzymes do not appear to have been quantified in ovine serum. The normal total enzyme level was found to be independent of time after lambing. Each of the 18 Scottish Halfbred ewes showed three isoenzymes of glucosephosphate isomerase which were designated GPI I, II and III, the former having the greatest anodic mobility.

The post-parturient fluctuations in absolute isoenzyme levels were difficult to interpret in view of the absence of information on the physiological significance of the multiple forms of this enzyme. Since no tissue shows a unique pattern of glucosephosphate isomerase isoenzymes except red blood cells (see Chapter 6, Part 2) the variations in isoenzyme levels with time after lambing cannot be attributed to release of the enzyme into the

serum from any particular tissue. Thus, the fluctuations in the absolute and percentage values of serum glucosephosphate isomerase isoenzymes with time after parturition were considered to be of little clinical significance.

B. NORMAL ISOENZYME LEVELS IN RAMS

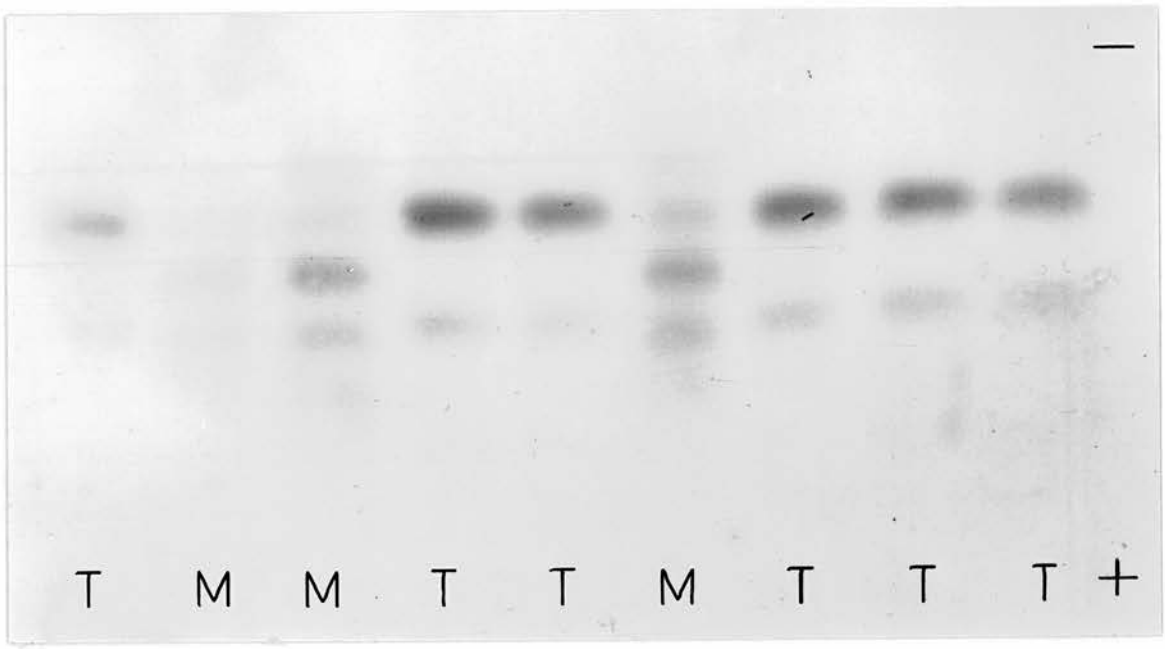
Nine adult Suffolk rams, four of which had been mated to the Halfbred ewes used in this experiment were each blood sampled on one occasion during the summer, when they were at pasture and were not working.

RESULTS

The mean values and standard deviations of total lactate dehydrogenase and its isoenzymes are shown in Table 6.46.

When glucosephosphate isomerase isoenzymes in the serum of the Suffolk rams were separated by electrophoresis, two distinct patterns were observed. Six of the nine animals showed the 3 band pattern which was observed in the Halfbred ewes, while the remaining three rams had a 5-6 band pattern. Bands were numbered from 1 to 6, the former having the greatest anodic mobility. The electrophoretic patterns obtained are shown in Fig. 6.2. Four of the nine rams were known to have been mated to the Halfbred ewes used in this experiment. Two of the four showed the 3 band and two the multiple band

Figure 6.2. Glucosephosphate isomerase isoenzyme variants in the serum of nine Suffolk rams. T = 3 band pattern, M = multiple band pattern.


—
T M M T T M T T T +

isoenzyme pattern.

The mean values and standard deviations for the total enzyme and isoenzyme levels were calculated for both isoenzyme patterns. The results are shown in Tables 6.47 and 6.48.

As in ewes, in rams with the 3 band pattern the greatest proportion of the glucosephosphate isomerase activity was shown by GPI III, followed by GPI II and GPI I. In rams showing the multiple band isoenzyme pattern, the greatest proportion of the activity was contributed by GPI 3, followed by GPI 2, GPI 4, GPI 1 and GPI 5, while GPI 6 showed the least activity and was only present in one individual.

DISCUSSION

The normal levels of total serum lactate dehydrogenase or its isoenzymes do not appear to have been previously reported specifically in rams. Comparisons between the total and isoenzyme levels in rams with those of ewes and lambs will be described in Part D (page 238).

Glucosephosphate isomerase isoenzymes formed two distinct electrophoretic patterns in the serum of the Suffolk rams, six individuals showing the 3 band pattern similar to that of the Halfbred ewes, while three rams showed a pattern of 5 or 6 bands. The existence of variants of this enzyme in the sheep

has been noted (Carter and Detter, unpublished observation, cited by Yoshida and Carter, 1969), but not described. The electrophoretic variants of this enzyme will be discussed in greater depth in Part 4 of this Chapter.

C. NORMAL ISOENZYME LEVELS IN LAMBS

VARIATION WITH AGE

RESULTS

The means and standard deviations were calculated for total serum lactate dehydrogenase and its isoenzymes in lambs sampled at birth, and at 2 weeks, 4 weeks and 10 weeks of age. Data from all four lamb groups (NL, EL, NH and EH) was used. The results, which are presented in Table 6.49, were obtained from the 69 lambs which were sampled on each of the four occasions. The results of the paired t-tests to assess the effect of age are presented in Tables 6.50 and 6.51.

In the case of glucosephosphate isomerase, Suffolk x Halfbred lambs, like the Suffolk rams, could be separated into two distinct populations according to their isoenzyme pattern. Out of a total of 124 lambs, 106 consistently showed the 3 band pattern, while the remaining 18 showed the multiple pattern. An additional band to the six demonstrated in ram serum was occasionally present. This band had a greater

anodic mobility than GPI 1, and was designated GPI 1a.

The mean values obtained for total glucosephosphate isomerase and its isoenzymes are presented in Tables 6.52, 6.53 and 6.54. Paired t-tests were carried out to determine the effect of age on the above parameters. The results for lambs with the 3 band pattern are shown in Tables 6.55 - 6.57. Fifty-five of the 106 lambs with the 3 band pattern and 13 of the 18 lambs with the multiple pattern were sampled on all four occasions. Only data from these lambs was used to calculate the means, standard deviations and t values. In addition, the total enzyme level was compared between all lambs with the 3 band and those with the multiple isoenzyme pattern by Student's t-test.

From Table 6.50, the mean total lactate dehydrogenase level was significantly lower at birth than in older lambs. This increase in the total enzyme level with age was associated with an increase in the absolute level of LDH_1 and LDH_2. The absolute levels of the other lactate dehydrogenase isoenzymes tended to fluctuate with age. When expressed as a percentage however, (Table 6.51) LDH_1 increased while LDH_4 and LDH_5 decreased with age after 2 weeks.

Unlike lactate dehydrogenase, total glucosephosphate isomerase was significantly higher at birth than at subsequent samplings (Table 6.55). The mean level in lambs with the 3 band pattern did not differ

significantly from those with the multiple isoenzyme pattern (figures not shown).

In lambs with the 3 band isoenzyme pattern, as in rams and ewes, GPI III showed the greatest activity followed by GPI II and GPI I in each age category. No clear pattern of changes in the absolute or percentage isoenzyme levels with age emerged (Tables 6.56 and 6.57) although many of the variations were highly significant.

In lambs showing the multiple pattern, minor fluctuations occurred with age, again without evidence of any trend ($p < 0.05$ in each case).

VARIATION WITH SEX

RESULTS

The results for both lactate dehydrogenase and glucosephosphate isomerase were calculated using data from all four groups of lambs (NL, EL, NH and EH) at birth and 10 weeks of age and are presented in Tables 6.58 - 6.63. The results of the Student's t-tests to compare the levels in male and female lambs are not presented since few significant differences were observed.

Total serum lactate dehydrogenase and glucose-phosphate isomerase did not differ significantly between male and female lambs at birth or at 10 weeks. At birth, percentage LDH_1 was significantly higher in female than male lambs ($p < 0.01$), but no difference was

observed in the absolute levels, nor in the absolute or percentage levels of glucosephosphate isomerase isoenzymes at either sampling.

VARIATION WITH GROWTH RATE

The object of the nutritional aspect of this experiment was to assess the effect on lamb survival and subsequent performance, of feeding two concentrate rations differing in undegradable protein content to ewes during late pregnancy, and to assess the effect of feeding bovine colostrum to lambs at birth, in addition to maternal colostrum. It was originally envisaged that the four different lamb treatments (see page 211) might produce a differential rate of growth in the lambs, and that the effect of growth rate on the normal total enzyme and isoenzyme levels of lactate dehydrogenase and glucosephosphate isomerase could be investigated by comparing the results obtained from each lamb group. It was first necessary to demonstrate the effect of lamb group on the rate of growth from birth to 2 weeks, 2 to 4 weeks and 4 to 10 weeks of age.

The mean and standard deviation of the daily weight gain in kg was calculated for each lamb group over each of the three time intervals mentioned above. One-way analysis of variance was carried out to test the null hypothesis that there was no difference in growth rate produced by lamb nutritional group. The means, standard deviations and F values are shown in Table 6.64.

The F values obtained were not significant at the 5% level and it was concluded that there was no evidence that lamb dietary group affected their growth rate at any of the three time intervals over which growth rate was estimated. Another possible means of grouping lambs according to growth rate was by litter size, regardless of nutritional group. By means of Student's t-tests, the rates of growth of singles, twins and triplets were compared at each of the three time intervals. At each time interval, singles had a significantly higher daily liveweight gain than twins or triplets ($p<0.01$) while the latter two groups did not differ significantly (data not shown). Thus lambs could be divided into only two growth rate groups (singles and twins/triplets) according to their litter size. Lambs were therefore divided into four arbitrary groups according to growth rate at each of the three time intervals (birth to 2 weeks, 2 to 4 weeks and 4 to 10 weeks), regardless of nutritional group or litter size. These groups were chosen to include a minimum of eight lambs, and were classified as follows:-

Group A - weight gain of <0.250 kg/day

Group B - " " " 0.250 - 0.299 kg/day

Group C - " " " 0.300 - 0.350 kg/day

Group D - " " " >0.350 kg/day

In the case of those showing the multiple glucosephosphate isomerase pattern in the serum, the lambs were divided into two groups, S and R - group S containing the 7 lambs showing the slowest, and group R the 8 lambs with the most rapid rate of growth because the number of lambs was insufficient to allow further subdivision. The mean daily weight gains of group S and R were as follows:

birth to 2 weeks:	group S,	0.208kg/day;
	group R,	0.360kg/day.
2 to 4 weeks:	group S,	0.309kg/day;
	group R,	0.403kg/day.
4 to 10 weeks:	group S,	0.294kg/day;
	group R,	0.363kg/day.

The rate of growth of lambs showing the 3 band pattern was compared with that of lambs showing the multiple glucosephosphate isomerase isoenzyme pattern and the results are shown in Table 6.65.

There was found to be no statistically significant difference in growth rate between lambs with different isoenzyme patterns and consequently, the data on the effect of growth rate on total glucosephosphate isomerase includes the values obtained from lambs with either isoenzyme pattern.

RESULTS

The mean values and standard deviations were

calculated for total serum lactate dehydrogenase and glucosephosphate isomerase and their isoenzymes in groups A, B, C and D at each of the three time intervals, and the results are shown in Tables 6.66 - 6.71. One-way analysis of variance was carried out on this data to determine whether the rate of growth during each time interval affected the levels of the above parameters at the end of that time interval. Where the analysis of variance provided evidence for differences in total enzyme and isoenzyme levels between groups, Student's t-test was carried out to determine between which groups significant differences were present. In the case of lambs showing the multiple glucosephosphate isomerase pattern, only Student's t-test was carried out since lambs were divided into two growth rate groups.

Total serum lactate dehydrogenase was significantly higher in group C lambs than in groups A and D ($p < 0.01$ and $p < 0.05$ respectively) during the period from birth to 2 weeks. This was reflected in the absolute LDH_4 level which was significantly higher in group C lambs than in groups A, B or D ($p < 0.001$, $p < 0.05$ and $p < 0.05$ respectively). The absolute LDH_5 level was significantly greater in group C than group A ($p < 0.01$). These differences were not observed when the isoenzyme values were expressed as a percentage, nor were they observed after 2 weeks of age.

Total serum glucosephosphate isomerase was significantly lower in group A lambs than in groups B, C and D ($p < 0.05$) at 2 weeks old. In lambs showing the 3 band pattern, this was associated with a significantly lower level of GPI III ($p < 0.05$), but the percentage isoenzyme levels did not vary with rate of growth. No differences were observed in glucose-phosphate isomerase or its isoenzymes according to growth rate from 2 to 4 or 4 to 10 weeks in lambs with the 3 band pattern, nor at any stage in lambs with the multiple pattern.

DISCUSSION

An appreciation of the effect of physiological parameters on total serum enzyme and isoenzyme levels is clearly necessary before their measurement can be of use for diagnostic purposes, but few such investigations have been undertaken in the sheep.

Age has been shown to affect total serum lactate dehydrogenase in Norwegian short-tailed lambs by Tollersrud and Baustad (1970) who reported that the level was high in young lambs with a peak at 12 hours after parturition, followed by a subsequent decline during the first 3 days of life. By 36 hours after birth, the activity had declined to a level below that observed immediately after birth. They suggested that the high level in neonates might be associated

with increased cell permeability due to an increase in adrenocorticotrophic hormone (ACTH), cortisone, insulin and catecholamine levels after birth resulting from the "stress" of transfer to a cold extra-uterine environment, increased physical activity and growth, or to rapid mobilisation of body fat reserves which are rich in lactate dehydrogenase (Hess, 1963). Healy and Falk (1974) in a study of Merino lambs found no significant difference in the total serum lactate dehydrogenase level from 1 to 18 days or between lambs of 2 weeks and 4 months of age.

My results indicated that the total lactate dehydrogenase level was significantly lower at birth than in older lambs. Since in my experiment lambs were first sampled at 24-36 hours, clearly any peak lactate dehydrogenase level at 12 hours as observed by Tollersrud and Baustad (1970), would not have been detected. This does not explain why a lower level was obtained at birth than in older lambs in the present experiment, a finding which is at variance with the observation by Healy and Falk (1974) that no significant change in total serum lactate dehydrogenase occurred from 1 to 18 days of age. The reason for this inconsistency is not clear, but may be due to breed differences. However, my observation that the lactate dehydrogenase level did not change from 2 to 10 weeks of age was consistent with

their finding that there was no significant difference between the level in lambs at 2 weeks and 4 months old.

The percentage values of lactate dehydrogenase isoenzymes in the serum of lambs at different ages have been reported by Tollersrud and Baustad (1970) and Tollersrud, Baustad and Flatlandsmo (1971). The former authors reported that the isoenzyme distribution showed only small changes from birth to 10 days with little tendency towards the isoenzyme pattern found in adult sheep.

My results for the percentage distribution of lactate dehydrogenase isoenzymes differed somewhat from those reported by Tollersrud and Baustad (1970) in that % LDH_5 was much higher in the present experiment with a mean value of 22.3% at birth and 22.9% at 2 weeks, compared with the complete lack of activity at approximately equivalent ages which had been previously reported. This discrepancy may be associated with breed differences or possibly with the loss of LDH_5 activity under the storage conditions used by these authors, LDH_5 being the most labile of the five isoenzymes. In the present experiment, there was a tendency for both the absolute and percentage values of LDH_1 to increase with age while the percentage of LDH_4 and LDH_5 decreased with age after 2 weeks. Thus from birth to 10 weeks, the isoenzyme pattern was changing towards

the adult pattern which, in agreement with the view of Tollersrud and Baustad (1970), was characterised by a higher percentage of LDH_1 (see Part D).

Several reports indicate that in most tissues, there is an increasing proportion of H subunits of lactate dehydrogenase (i.e. an increase in the anodic isoenzymes) with increasing gestational age (Hinks and Masters, 1964; Davis et al., 1973) and that these pre-natal changes tend to continue after birth (Courtney and Ebron, 1978; Walden and Schiller, 1980). Since cellular enzymes are liberated into the serum during the normal turnover of cells (Mattenheimer, 1971), it would be expected that the serum isoenzyme patterns at a particular age would reflect the isoenzyme complement of the tissues at that age. This would provide an explanation for the age-related increase in the percentage of LDH_1 and decrease in LDH_4 and LDH_5 observed in the present experiment.

Changes in total serum glucosephosphate isomerase or its isoenzymes in relation to age do not appear to have been described in any species.

The total glucosephosphate isomerase level in contrast to lactate dehydrogenase, was significantly higher at birth than in older lambs. Glucosephosphate isomerase is known to be particularly active in rapidly dividing cells, rendering its measurement in serum a useful diagnostic aid in neoplastic disease in man (Schwartz and Bodansky, 1966; Munjal and

Brady, 1978; Munjal, 1980). The rapid rate of cell division in neonates may thus provide one explanation for the higher total serum glucosephosphate isomerase level at 24-36 hours of age. A second possibility is the hormone-induced increase in cell membrane permeability and rapid mobilisation of fat reserves in neonates, leading to an increased serum enzyme level (Hess, 1963), but if this were the case serum lactate dehydrogenase would also be expected to show a higher level soon after birth. The release of glucosephosphate isomerase into the serum from skeletal muscle as a result of trauma during parturition is a less likely source since again, this would be expected to result in a higher level of serum lactate dehydrogenase at birth.

Like the Suffolk rams, lambs showed one of two distinct glucosephosphate isomerase isoenzyme patterns, 85.5% having the 3 band and 14.5% the multiple band pattern. When the changes in the absolute and percentage values of the isoenzymes were assessed in relation to age in lambs showing the 3 band pattern, the absolute isoenzyme levels tended to follow the changes in total enzyme level, with higher levels at birth but the percentage values showed no consistent pattern of changes. In lambs showing the multiple pattern, minor variations were observed in relation to age, but again no distinct trend was apparent.

In the absence of information on the physiological significance of the multiple forms of glucosephosphate isomerase, the age-related variations are difficult to interpret, but in any case, the changes did not show any clearcut trend.

Sex as a physiological factor affecting serum enzyme levels was investigated by Healy and Falk (1974), who found no significant difference between total serum lactate dehydrogenase levels in male and female lambs.

Similar results have been reported in calves (Bide, Bowden and Tumbleson, 1977). The percentage of LDH_1 in the serum of young women is higher than in men, a finding which has been attributed to the higher circulating oestrogen levels in women (Cohen, Block and Djordjevich, 1967), while Frahm et al. (1978) reported that bulls had a significantly higher total plasma lactate dehydrogenase level than cows. The effect of sex on normal serum lactate dehydrogenase isoenzyme levels has apparently not been described in the sheep.

In this experiment, neither total serum lactate dehydrogenase nor the absolute isoenzyme levels were significantly different in male and female lambs either at birth or at 10 weeks of age. The percentage value of LDH_1 however, was significantly higher in female than male lambs at birth ($p < 0.01$) but little importance can be attached to this observation

since the difference was not significant at 10 weeks, and it was unlikely to be due to differences in circulating oestrogen levels in one day old lambs.

Sex related differences in total serum glucose-phosphate isomerase and its isoenzymes have not been investigated in the domestic animals. In the present study, sex did not affect either total glucosephosphate isomerase or its isoenzyme levels in lambs with either isoenzyme pattern at birth or at 10 weeks of age.

Growth rate in relation to serum enzyme levels in all species is poorly documented. Healy and McInnes (1975) in a study of various biochemical parameters in relation to liveweight gain in lambs, found that in four groups of eight-month old Merino and Merino cross lambs with mean liveweight changes of +0.205, +0.163, +0.037 and -0.046kg/day, no significant difference in total plasma ornithine carbamoyl transferase, aspartate aminotransferase, creatine kinase or lactate dehydrogenase occurred. Baker and Manwell (1977) reported that Merino and Merino cross lambs which were heterozygotes for variants of the NADP-dependant dehydrogenases grew an average of 10.4% faster than homozygotes. Other reports on total enzyme or isoenzyme levels in relation to rate of growth in any species appear to be lacking.

In my experiment, significant differences in total serum lactate dehydrogenase were only observed in lambs at 2 weeks of age, according to their rate of growth during the period from birth to 2 weeks, with the lowest level being observed in lambs with the lowest and highest daily liveweight gains. This lower total enzyme level was associated with lower absolute levels of LDH_4 in groups A and D and LDH_5 in group A, but the percentage distribution of the isoenzymes did not change significantly during this period. Total serum glucosephosphate isomerase also varied with growth rate from birth to 2 weeks, but not during the two subsequent time intervals. Lambs growing at the slowest rate had the lowest total serum enzyme level. In lambs with the 3 band glucosephosphate isomerase isoenzyme pattern, the lower total enzyme level was associated with a lower absolute GPI III value, which just reached statistical significance, but in lambs with the multiple pattern neither the absolute nor percentage values were affected by rate of growth.

Since total serum lactate dehydrogenase, LDH_4 and LDH_5 were significantly higher in lambs with an intermediate rate of growth, there was no clear relationship between these parameters and liveweight gain and the reason for the higher levels in group C lambs is unknown.

Similarly, the reason for the lower absolute GPI III value is not clear since glucosephosphate isomerase isoenzymes do not show a tissue-specific pattern (see Part 2) and their physiological function is uncertain.

It was noticable that where significant differences were observed in total or isoenzyme levels, they occurred only during the first 2 weeks after birth. When mean daily weight gain was expressed as a percentage of mean liveweight of lambs of all four growth rate groups at 2 weeks, 4 weeks and 10 weeks of age, the values obtained were 3.1%, 2.6% and 1.2%, respectively. Thus, statistically significant differencesin total and isoenzyme levels associated with differencesin daily liveweight gain were only observed when the rate of growth in relation to liveweight was maximal.

The observations of Baker and Manwell (1977) that lambs showing genetic variants of the NADP-dependant dehydrogenases had significantly different rates of liveweight gain prompted an investigation into the daily weight gains in lambs with the 3 band and lambs with the multiple glucosephosphate isomerase isoenzyme pattern. Although the mean liveweight gains were higher during each of the three time intervals over which growth was measured in lambs showing the multiple pattern, the differences were not statistically

significant.

When considered in the light of their clinical rather than their statistical significance, the observations of the greatest practical importance were the change with increasing age in lambs in total serum lactate dehydrogenase and glucosephosphate isomerase and the trend with age towards the adult lactate dehydrogenase isoenzyme pattern.

D. COMPARISON BETWEEN NORMAL LEVELS IN RAMS, EWES AND LAMBS

It was of interest to determine whether the total and isoenzyme levels of lactate dehydrogenase and glucosephosphate isomerase in rams, ewes and lambs differed since any such differences would be of importance in the interpretation of results for diagnostic purposes.

RESULTS

Student's t-test was carried out to compare the values for the above parameters in the 17 Halfbred ewes which were sampled at 10 weeks after lambing (one of the 18 ewes died between the first and second samplings) with those of rams. The 10 week sampling of the ewes was chosen for comparison since this sampling closely approximated to the time of year that the rams were sampled, thereby eliminating any effect

of season which has been shown to affect total serum lactate dehydrogenase and its isoenzymes in cattle (Roussel and Stallcup, 1967). Similarly, the mean values obtained from all lambs at birth and at 10 weeks of age were compared with the values in non-gravid (10 week post-parturient) ewes and with rams. It was not possible to compare glucosephosphate isomerase isoenzyme levels in rams and lambs showing the multiple band pattern since only 3 rams showed this variant.

The results of the statistical comparisons between the values in lambs with those in rams and ewes are presented in Tables 6.72 - 6.75.

The mean total serum lactate dehydrogenase level in rams was not significantly different from that of the 10 week post-parturient ewes. A comparison between the absolute isoenzyme levels in rams and ewes showed that ewes had a significantly higher LDH_2 level ($p < 0.001$) while rams had a significantly higher LDH_5 level ($p < 0.05$). These differences were also observed when the isoenzyme levels were expressed as a percentage of the total activity. Differences between the mean levels of LDH_1, LDH_3 and LDH_4 in rams and ewes were not significant.

The mean total serum glucosephosphate isomerase levels in rams was significantly higher than the mean value in 10 week post-parturient ewes ($p < 0.01$).

This was associated with a higher absolute level of all three isoenzymes in rams showing the 3 band pattern.

The mean total serum lactate dehydrogenase was significantly higher in 10 week old lambs than in adult sheep, but this difference was not observed in lambs at birth. The absolute and percentage levels of LDH_1 were lower in both ages of lambs than in adult sheep while the levels of LDH_2, LDH_3, LDH_4 and LDH_5 tended to be higher in lambs.

Total serum glucosephosphate isomerase was significantly higher in lambs of both age groups than in ewes but not rams. The higher total enzyme level in lambs than ewes was associated with a greater activity of all three isoenzymes at birth, and a greater activity of GPI II and III at 10 weeks old. When the percentage levels of the isoenzymes were compared, no statistically significant difference was observed between lambs at birth and ewes, since the higher total enzyme level in the lambs was associated with an absolute increase in all three isoenzymes. However, minor differences were observed between the percentage levels in ewes and in lambs at 10 weeks. The glucosephosphate isomerase isoenzyme distribution in rams with the 3 band pattern was not significantly different from that of lambs of either age.

DISCUSSION

Total serum lactate dehydrogenase activity is reported to be higher in lambs (of up to 10 days and 4 months old respectively) than in adult sheep (Tollersrud and Baustad, 1970; Healy and Falk, 1974). The results of the present experiment confirm this view although the difference was not significant at birth, since lambs of this age had a significantly lower total enzyme level than older lambs (see page 223). When the isoenzyme levels in lambs were compared with the levels in rams and ewes, lambs at birth and 10 weeks old showed markedly lower absolute and percentage values of LDH_1 than adult sheep and a tendency towards higher levels of LDH_2, LDH_3, LDH_4 and LDH_5. The reason for the differences in isoenzyme pattern can be explained by the fact that there is an increasing proportion of H subunits in the tissues (i.e. an increasing percentage of the anodic lactate dehydrogenase isoenzymes) with age to maturity in animals, the serum isoenzyme pattern reflecting this changing tissue isoenzyme pattern. This concept is discussed more fully in Chapter 2 and on page 232 of this Chapter.

The results of the comparison between total serum lactate dehydrogenase levels in rams and ewes in the

present experiment indicated that, as in humans (Cohen, Block and Djordjevich, 1967), the level did not differ significantly between adult males and females. However, differences were observed in the isoenzyme pattern - LDH_5 was significantly higher and LDH_2 significantly lower in rams. While it could be proposed that the higher serum LDH_5 level in rams might be associated with the greater muscle mass in male than female sheep (Hafez and Dyer, 1969), the sex differences were treated with caution since they might also be attributed to breed differences.

Differences in total serum glucosephosphate isomerase and its isoenzymes in adult and young animals do not appear to have been previously described. Lambs had a significantly higher total serum glucose-phosphate isomerase level than ewes, but not rams, and rams had a significantly higher level than ewes. The higher total enzyme level in lambs than ewes may be associated with a more active glycolytic pathway in the former, but the lack of significant difference between the levels in lambs and rams is difficult to reconcile with this view. The higher total enzyme level in lambs and rams than ewes was associated with a higher absolute level of all three, rather than any particular isoenzyme in those showing the 3 band pattern.

In Part D, the findings of potential importance

in the interpretation of serum enzyme and isoenzyme estimations for clinical purposes were the marked differences in total lactate dehydrogenase and glucosephosphate isomerase and lactate dehydrogenase isoenzyme levels between lambs and adult sheep.

CONCLUSIONS FROM PART 3

The levels of total serum lactate dehydrogenase and glucosephosphate isomerase and their isoenzymes were established in clinically normal rams, ewes and lambs. Changes in the lactate dehydrogenase pattern were observed after lambing in ewes and were consistent with an increase in gluconeogenesis.

Lambs showed lower levels of LDH_1 and higher levels of the more cathodic lactate dehydrogenase isoenzymes than adult sheep, with a trend towards the adult pattern from 2 weeks after birth. This was consistent with the changing tissue isoenzyme distribution with age. Glucosephosphate isomerase isoenzymes showed no clear age-related trend.

In rams and lambs, two distinct glucosephosphate isomerase isoenzyme patterns were observed but no conclusions could be drawn regarding the genetic basis of the transmission of this variant since the paternity of the lambs was unknown.

In lambs, sex-related differences in lactate

dehydrogenase isoenzymes were minor, while no such differences were observed for glucosephosphate isomerase.

The total level of lactate dehydrogenase in the serum was lowest in the slowest and fastest-growing lambs whereas total glucosephosphate isomerase was lowest in the slowest-growing lambs. The relationship between rate of growth and the total and isoenzyme levels of lactate dehydrogenase and glucosephosphate isomerase was not clear but where significant differences were observed, they occurred during the period from birth to 2 weeks when daily liveweight gain as a percentage of liveweight, was maximal. Lambs with the multiple glucosephosphate isomerase pattern showed a similar daily liveweight gain to those with the 3 band pattern.

It is essential that the statistically significant differences described should be considered in the light of their clinical significance. The findings which were considered to be of importance in the interpretation of results for diagnostic purposes are as follows:

(a) Total lactate dehydrogenase and its isoenzymes in IU/l vary with time after parturition in ewes.

(b) Total lactate dehydrogenase and glucosephosphate isomerase levels, and lactate dehydrogenase isoenzymes change with age in lambs and differ between lambs and adult sheep.

Changes associated with sex and growth rate were minimal and of little practical importance.

PART 4 - VARIANTS OF GLUCOSEPHOSPHATE ISOMERASE

INTRODUCTION

During the previous experiment (Chapter 6, Part 3), it was observed that in Scottish Halfbred ewes, serum glucosephosphate isomerase isoenzymes were separated into three distinct bands, whereas three out of nine Suffolk rams and 14.5% of Suffolk x Halfbred lambs showed a distinct isoenzyme pattern of 5-7 bands. The allelozymes of glucosephosphate isomerase have been extensively studied in man and to a lesser extent in the domestic animals, but apparently not in the sheep. The aim of this experiment was to determine the frequency of the two glucosephosphate isomerase alleles in the progeny of rams and ewes of known phenotype, and to investigate the inheritance of the two patterns.

EXPERIMENTAL DESIGN

Twenty-three Halfbred ewes showing the 3 band pattern were mated to the three Suffolk rams from the previous experiment which showed the multiple band glucosephosphate isomerase isoenzyme pattern.

MATERIALS AND METHODS

Serum samples were collected as previously described. Glucosephosphate isomerase isoenzymes were separated by polyacrylamide gel electrophoresis and visualised by the tetrazolium method as discussed in

Chapter 4. The migration distance of bands was measured in samples showing the different patterns which were run adjacent to one other in the gel.

It was originally intended to collect tissue samples from selected lambs at slaughter to determine whether their tissues showed the same isoenzyme pattern as the serum but unfortunately the lambs were sold before slaughter and it was only possible to obtain a sample of red blood cells from one lamb.

RESULTS

All ewes showed the 3 band glucosephosphate isomerase electrophoretic pattern, (variant T) and all three rams showed the multiple band pattern (variant M). Of their 32 progeny, 23 showed the 3 band pattern (71.9%) and 9 showed the multiple pattern (28.1%) which was identical to that of the rams (Table 6.76). The one lamb in which the red blood cell isoenzyme pattern was investigated showed the multiple pattern in both serum and red cells.

Fifteen ewes produced single lambs, seven produced twins and one had triplets. In four sets of twins, both lambs showed the 3 band pattern while in the remaining three sets, one lamb showed the 3 band and one the multiple pattern. All three members of the set of triplets showed the multiple pattern. In lambs showing the 3 band pattern, 8 were male and 13 female (the sex of two lambs was not recorded) and

in lambs showing the multiple pattern, 5 were male and 4 female. The proportions of male and female lambs showing each pattern were shown not to be significantly different from a ratio of 1:1 by the Chi-squared goodness-of-fit test.

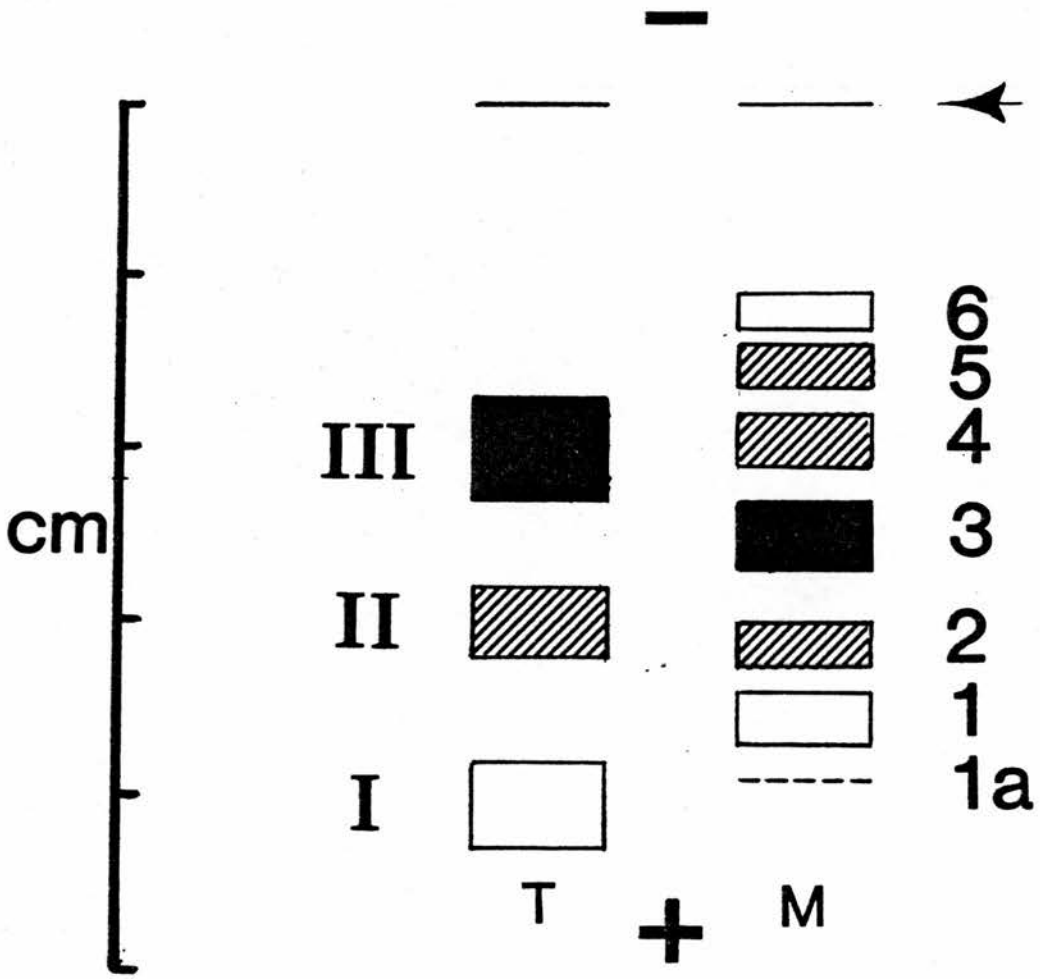
TABLE 6.76.

INHERITANCE OF GLUCOSEPHOSPHATE ISOMERASE TYPES IN 23 MATINGS

	NUMBER AND TYPE OF OFFSPRING			
	T		M	
Matings	no.	frequency	no.	frequency
T x M	23	0.72	9	0.28

In order to ascertain whether the observed ratio of 1:2.6 for lambs showing the multiple and 3 band patterns respectively was significantly different from a 1:1, 1:2, 1:3, 1:4, 1:5 or 1:6 ratio, Chi-squared goodness-of-fit tests were carried out. The observed ratio was significantly different from ratios of 1:1 and 1:6 ($p < 0.05$) but not from ratios of 1:2, 1:3, 1:4 and 1:5. Measurement of distance of migration from the origin for each band showed that GPI 4 corresponded in migration distance to GPI III, while GPI 2 overlapped with GPI II. When detectable, GPI 1a corresponded to GPI I, although the latter was much wider. The results are shown diagrammatically in Fig.6.3.

Figure 6.3. Diagrammatic representation of the T and M variants of glucosephosphate isomerase showing the relative migration distances and intensity of staining of the bands. Arrow indicates origin.


cm
III
II
I
T
M
6
5
4
3
2
1
1a

DISCUSSION

Genetically determined variants of glucosephosphate isomerase are known to occur in man, Rhesus monkeys, mice, rabbits, cats, pigs and horses. Variants are usually described in red cell lysates although they have been demonstrated in all tissues of an individual animal (Welch, Fitch and Parr, 1970). This view is consistent with the identical pattern which was observed in the serum and red cells of one lamb.

Carter and Parr (1967) reported two variants in the mouse, each with single isoenzymes differing in electrophoretic mobility, while the progeny of two such mice showed both bands present in the parents plus an additional band of intermediate mobility. Similar findings have been reported in the rabbit (Welch, Fitch and Parr, 1970). The latter authors considered that the parents were homozygous for the variants which they showed, while their progeny, all of which showed a 3 band pattern, were heterozygotes. When two heterozygotes were mated, offspring showing both homozygous variants and the heterozygous variant were produced. Since the enzyme is a dimer, the heterozygotes were assumed to possess a hybrid enzyme (i.e. the intermediate band present in neither parent) containing a subunit from each allele.

In the pig, Saison and O'Reilly (1971) demon-

strated two variants, A and B, each with a distinct multiple band pattern, and a third type, AB which showed all the bands of variants A and B. The results were compatible with a two allele system with codominant inheritance i.e. both alleles were fully expressed in the heterozygote. In the horse, four variants were detected in each of two Swedish breeds (Sandberg, 1973). This was attributed to the existence of three autosomal alleles, F, I and S which gave rise to the four variants - F, I, FI and IS. The homozygotes F and I each showed one band of differing electrophoretic mobility while FI showed the F and I bands, plus a third of intermediate mobility and IS showed the I and S bands plus an intermediate band. In Rhesus monkeys, VandeBerg and Stone (1978) described similar results to those reported by Saison and O'Reilly (1971) in the pig, with two homozygous and one heterozygous variant controlled by two codominant autosomal alleles. In Australian domestic cats, three multiple band patterns occur, designated F, FS and S, type FS showing all bands observed in both F and S. This polymorphism was also considered to be controlled by two codominant autosomal alleles (Auer and Bell, 1980).

In the present experiment, the results cannot be explained by simple Mendelian inheritance since if T (or M) were dominant, all progeny of T x M matings

would be phenotypically T (or M), assuming that both variants were homozygous. If individuals showing the M variant were heterozygous (Mt), M being dominant to t and those showing the T variant were homozygous (tt), 50% of the progeny would be phenotypically M and 50% would be phenotypically T. The results obtained were inconsistent with a 1:1 ratio, however.

The fact that two bands of the 3 band pattern corresponded to, and the third band overlapped with bands of the multiple pattern suggested the possibility that the M variant represents a heterozygote and the T variant a homozygote, the variants being controlled by two codominant alleles, as in other species. If this were the case, a second homozygote should exist showing bands GPI 1, GPI 3, GPI 5 and GPI 6. However, in this experiment involving 58 sheep, and in the previous experiment involving 151 sheep of the Suffolk, Halfbred and Suffolk x Halfbred types, no such individuals were detected. Consequently, there was no evidence that the variants are controlled by two codominant alleles. Although the findings can be explained on the basis of incomplete penetrance (i.e. certain individuals of a specific genotype do not express the phenotype normally associated with that genotype), this is a somewhat vague concept which is often applied when the data are not compatible with any known means of transmission.

Thus, the results do not elucidate the mechanism of transmission of the electrophoretic variants of glucosephosphate isomerase, but several important factors have been observed regarding the nature of this mechanism:

(a) The variants are not controlled by simple Mendelian inheritance.

(b) There was no evidence that the variants were controlled by codominant alleles since only one homozygote (type T) was detected.

(c) The results were compatible with a 1:2, 1:3, 1:4, or 1:5 ratio but not with a 1:1 or 1:6 ratio of M to T phenotypes.

(d) Neither variant appeared to be sex-linked.

The existence of the M variant can, however, be considered as evidence of true polymorphism of glucose-phosphate isomerase in the sheep since the frequency (0.28) was higher than the arbitrary values of 0.001 or sometimes 0.005 used to differentiate polymorphism from monomorphism. Although this figure for the frequency in the lambs is artificially high, since the sires were selected to show the multiple pattern, the figure obtained in the previous experiment when rams were not selected (0.14) was still indicative of true enzyme polymorphism. There are no previous published reports of the polymorphism of this enzyme in the sheep.

CHAPTER 7

ENZYME ESTIMATIONS IN DISEASE

Elevated serum levels of enzymes and isoenzymes may result from the following (after Zimmerman and Seeff, 1970):

1. increased release from tissues
   (a) necrosis
   (b) increased cell membrane permeability without necrosis
2. increased tissue source of enzymes
3. impaired excretion

The rate of release of an enzyme from tissues depends on the normal enzyme content of the damaged tissue, the type and extent of the damage and the intracellular site of the enzyme, since binding to cell structures will impair release. Once present in the circulation, the serum half-life of the enzyme, which is dependant on the rate of inactivation and degradation by the reticulo-endothelial system or on the rate of excretion via the kidneys or in the bile, determines the time-span over which increased serum levels will be detectable. Inactivation and degradation is the means of elimination of most cellular enzymes from the serum but a few enzymes with molecular weights of less than 70,000 daltons are excreted in the urine while hepatic alkaline phosphatase in man is excreted via the bile.

In general, cellular enzymes which are released into the serum as a result of necrosis or increased cell membrane permeability will show the greatest elevations in the acute phase of the disease when these two processes are most active and the serum half-life is such that the levels have not yet returned to normal, but elevations in the chronic phase may be detected when the cellular damage is still active. Thus, the greatest proportion of the literature on the use of enzymes in diagnosis describes serum or plasma enzyme changes in acute disease.

Serum enzyme and isoenzyme estimations are relatively reliable in the detection of acute or progressive and active damage to skeletal muscle, heart and liver in most species.

In acute or progressive skeletal muscle damage, serum enzyme determinations are generally preferable to an invasive technique such as muscle biopsy for histology, muscle creatine and creatinine estimations or to creatine, creatinine and myoglobin measurements in urine, which may be difficult to obtain, while haematological examination is rarely of use. A number of serum enzymes have been employed in acute skeletal muscle disease such as trauma, progressive muscular dystrophy in man, paralytic myoglobinuria in horses, nutritional muscular dystrophy in lambs

and calves and the porcine stress syndrome. The enzymes used vary little between the species although the results obtained may differ. The most commonly used are creatine kinase and its isoenzymes, lactate dehydrogenase and its isoenzymes, aspartate aminotransferase and aldolase. In progressive muscular dystrophy in man for example, total serum creatine kinase and lactate dehydrogenase are elevated with an increase in percentage of LDH_1 and LDH_2 (Yasmineh et al., 1978). In acute nutritional muscular dystrophy in lambs and calves however, the percentage of LDH_5 is increased (Boyd, 1964 ; Dotta and Pellegrino, 1972). Boyd (1964) considered that the total enzyme and isoenzyme levels were related to the extent and severity of the muscle degeneration. Serum enzyme increases in acute skeletal muscle disease are often very large and therefore easily detectable. Paulson, Pope and Baumann (1966) for example, observed a 645% and 2941% increase in serum lactate dehydrogenase and aspartate aminotransferase, respectively, and increases in the lactate dehydrogenase isoenzymes of 294% (LDH_1), 447% (LDH_2), 705% (LDH_3), 1200% (LDH_4) and 9000% (LDH_5) in nutritional muscular dystrophy in lambs. Creatine kinase is considered to be the most sensitive indicator of acute skeletal muscle damage and Tollersrud (1971) noted an increase of approximately

26,000% in the serum of dystrophic lambs.

Acute myocardial damage occurs most commonly in man. Several serum enzymes increase after myocardial infarction, including aspartate aminotransferase, creatine kinase, lactate dehydrogenase, malate dehydrogenase and glucosephosphate isomerase. None of these enzymes when measured as the total enzyme level are specific to the myocardium, and creatine kinase and lactate dehydrogenase isoenzyme estimations are of value in distinguishing skeletal muscle from heart muscle damage. The presence of creatine kinase MB in the serum concurrently with a raised total enzyme level or an increase in the LDH_1: LDH_2 ratio with or without a raised total level are almost diagnostic of myocardial infarction in the presence of the appropriate clinical signs (Papadopoulos, 1981; Lang and Würzburg, 1982). Similar changes in lactate dehydrogenase and its isoenzymes occur in myocardial damage in the domestic animals (Dotta and Pellegrino, 1972; Bezzecchi et al., 1979) but creatine kinase isoenzyme estimations for this purpose do not appear to have been described except in laboratory animals.

A large number of liver function tests other than serum enzyme estimations are available including serum bilirubin, urinary and faecal bile pigments, dye excretion tests, serum protein determinations, prothrombin times, blood ammonia, and various protein,

lipid and carbohydrate metabolism tests. Radiography of the liver is impracticable in ruminants and horses. In ruminants, serum bilirubin levels are only raised in very severe liver damage, while serum proteins and prothrombin times are non-specific and blood ammonia, dye excretion tests and metabolism tests difficult and time-consuming to perform. "Liver-specific" enzyme determinations, however, are considered to be the most sensitive and reliable tests available for hepatic necrosis (Cornelius, 1970).

Marked species differences exist in the enzyme complement of the liver with resultant differences in the enzymes which are released into the serum in acute hepatic disease. In each species, several enzymes or their isoenzymes are considered to be reasonably liver-specific, particularly when two or more are measured simultaneously. In man for example, alanine aminotransferase (Zimmerman and Seeff, 1970), alkaline phosphatase plus gamma-glutamyl transpeptidase (Betro and Edwards, 1973), glutamate dehydrogenase (Lieber, Shaw and Van Waes, 1978), lactate dehydrogenase isoenzymes (Wieme and Van Maercke, 1961) and alkaline phosphatase isoenzymes (Rhone, White and Gidaspow, 1973; Burlina and Bugiardini, 1978) are almost liver-specific. Alanine aminotransferase is fairly liver-specific in dogs and cats, alkaline phosphatase isoenzymes in dogs, sorbitol dehydrogenase and glutamate

dehydrogenase in ruminants (Boyd, 1962; Ford, 1967) and sorbitol dehydrogenase in horses. Gamma-glutamyl transpeptidase, although not liver-specific, is useful in conjunction with other enzyme estimations in man and dogs and because of its long serum half-life, is useful in following the course of liver disease. In sheep, it is increased in cholestasis and bile duct damage but not hepatocellular necrosis (Ford, 1974).

From the foregoing, it may be concluded that the detection of damage to skeletal muscle, myocardium and liver by serum enzymology is a fairly well established technique and, in comparison with the detection of damage to other organs, relatively easy. However, enzyme tests have also been employed in the diagnosis of diseases affecting bone, central nervous system and kidney.

In diseases of bone, serum enzyme estimations are of secondary importance to radiology, serum calcium and serum inorganic phosphate. Enzyme measurements are practically confined to alkaline phosphatase and its isoenzymes since this enzyme is involved in osteogenesis. In man, alkaline phosphatase and its isoenzymes have been studied in conditions such as fractures, rickets, multiple epiphyseal dysplasia, osteomalacia, primary bone tumours and Paget's disease (Nagamine and Ohkuma, 1975; Burlina

and Galzigna, 1976; Siede and Seiffert, 1977). In domestic animals, there are few such reports. Olson et al. (1958) found no correlation between fluoride ingestion and serum alkaline phosphatase in cattle but Healy (1971) reported total enzyme and iso-enzyme changes in phosphorus deficiency in the same species.

In diseases of the central nervous system, haematological findings are non-specific and it may be necessary to resort to cerebro-spinal fluid analysis. Serum enzyme estimations are often of limited value except in severe cases, in view of the inability of most enzymes to cross the intact "blood-brain barrier". In human medicine, serum aspartate aminotransferase has been shown to be elevated in cerebral infarction (Junger and Gunnar, 1957) but clearly this is not a specific test. Creatine kinase isoenzyme BB, which is the predominant iso-enzyme in nervous tissue, increases in the serum after brain surgery (Nealon and Henderson, 1975b), but in generalised convulsions, skeletal muscle is the source of raised serum levels. In sheep, serum creatine kinase increases in polioencephalomalacia and focal symmetrical encephalomalacia (Smith and Healy, 1968).

In renal disease, urine analysis and blood urea estimations are by far the most important diagnostic

aids. Regarding enzyme estimations, the bone isoenzyme of alkaline phosphatase and creatine kinase BB are said to increase in chronic renal failure in man (Skillen and Pierides, 1977; Chuga and Bachner, 1978). Gamma-glutamyl transpeptidase, although active in kidney, tends to be excreted in the urine in kidney disease and increased blood levels are rarely observed (Rosalki, 1975). Similar findings have been reported for gamma-glutamyl transpeptidase in sheep (Ford, 1974; Shaw, 1976).

The extent of damage to the respiratory system is very difficult to assess in the intact animal. Haematological changes are non-specific, even in bacterial pneumonia, and in ruminants such changes may only be evident in severe disease. Radiography may indicate pneumonia, tumours and hydro-,pneumo- or haemothorax, but this technique is not practicable in pigs, ruminants and horses. Bacteriological examination of nasal swabs in suspected bacterial pneumonias is not always reliable since many potentially pathogenic bacteria are present in the normal animal. Attempts at diagnosing viral pneumonias may be made by the demonstration of rising serum antibody titres e.g. serum neutralisation test for infectious bovine rhinotracheitis and enzootic pneumonia of calves, and demonstration of the causal virus by immuno-fluorescence is of use in conjunction with

rising antibody titres but no information is gained regarding the extent of the tissue damage. Virus pneumonias are more reliably diagnosed by postmortem and histological findings. In parasitic respiratory disease, the presence of lungworm larvae (Dictyocaulus filaria and Muellerius capillaris in sheep) in the faeces is diagnostic in the presence of appropriate clinical signs, but the larvae may not be detected and repeated examination is often necessary, while clinical signs may not be evident. The examination of pleural effusions may be performed including macroscopic examination, specific gravity, protein content, microscopic examination and bacteriology, but in many cases, the results are not clearcut and re-examination may be required. However, techniques such as haematology, immunology, virus detection and demonstration of larvae are of little or no value in the detection of residual lung damage in chronic respiratory disease. The existence of a specific and rapidly-performed test for the detection of chronic pneumonia and for the assessment of the severity of lung damage in both acute and chronic pneumonia would have considerable diagnostic implications, particularly the ability to pin-point chronic pneumonia as a cause of unthriftiness in growing ruminants. Such an aim might be achieved by the study of selected enzymes and their isoenzymes.

Attempts to detect lower respiratory tract disease by serum enzymology in man have been largely unsuccessful. Keiding (1974) noted an increase in one alkaline phosphatase isoenzyme in various lung diseases in man but this change was not specific to lung disease. Similarly, the appearance of an "atypical" creatine kinase band was reported in pulmonary disease patients but also in patients with chronic heart disease (Sax et al., 1979). An increase in the BB isoenzyme has been reported in the serum of 39% of patients with extensive lung carcinomas but not at an earlier stage in the disease (Gazdar et al., 1981). In domestic animals, an increase in total serum lactate dehydrogenase and the development of an abnormal isoenzyme pattern has been described in pigs with bronchopneumonia (Georgiev and Monov, 1976) and horses with acute pulmonary emphysema (Ottonello, Ubaldi and Corbella, 1979). These findings appeared to have some diagnostic potential in these species, but similar investigations in ruminants have not been undertaken.

The detection of damage to the gastrointestinal tract is equally difficult to assess in the living animal. Bacterial diseases such as colibacillosis and salmonellosis require bacterial isolation from faecal swabs followed by serotyping, but false negative and false positive results frequently occur.

In viral diseases such as transmissible gastro-enteritis in pigs, virus isolation and serum neutralisation tests may be attempted.

The diagnosis of parasitic infestations by faecal egg counts suffers from a number of disadvantages. Firstly, the significance of the number of eggs present depends on the species of nematode and species identification from egg morphology is in many cases difficult. Secondly, the number of eggs in the faeces gives little indication of the worm burden. Thirdly, in certain diseases such as Nematodirus battus infection and ostertagiasis, damage is caused by the larvae and therefore egg counts will be of little use. Arbitrary figures for the number of eggs of different parasites which are regarded as being significant have been reported, but clinical response is influenced by age and intercurrent disease. Haematology is generally unhelpful except in determining the severity of blood loss caused by parasites such as Haemonchus contortus. Thus, the present position regarding the detection of both acute and chronic damage to the gastrointestinal tract from a variety of causes is somewhat unsatisfactory. The only enzyme which is used routinely to detect damage to the digestive tract is serum pepsinogen. In parasitic gastritis in cattle and sheep serum pepsinogen levels may be elevated, providing a means of detecting larval

damage, but high levels are only observed during the acute phase of the disease, and normal individuals sometimes show raised levels.

Changes in the serum levels of a number of cellular rather than secreted enzymes have been described. Qirbi and Moss (1975) reported the occasional appearance of an abnormal alkaline phosphatase isoenzyme in ulcerative colitis and Crohn's disease in man, while in gastric and colonic cancer, glucosephosphate isomerase may increase (Munjal and Brady, 1978). "Atypical" creatine kinase has been detected in gastrointestinal disease in man (Sax et al., 1979) but this finding is not pathognomonic. Increases in total serum creatine kinase and lactate dehydrogenase and changes in their isoenzyme levels occur in colonic infarction in the dog (Graeber et al., 1981). In intestinal parasitism in pigs, total serum alkaline phosphatase decreased (Enigk, Dey-Hazra and Dimitrov, 1973) whereas in cattle with intractable diarrhoea and horses with intestinal parasitism and diarrhoea, "intestinal" alkaline phosphatase was elevated (Healy, 1971; Blackmore and Palmer, 1977). In sheep, a number of reports describe changes in the activity of the intestinal brush border enzymes in intestinal parasitism (Coop, Mapes and Angus, 1972; Mapes and Coop, 1973) but serum enzyme measurements were not undertaken.

CONCLUSIONS

Serum enzyme and isoenzyme measurements are relatively reliable diagnostic aids in the detection of skeletal muscle, heart muscle and liver damage in a variety of species. In diseases of bone, central nervous system and kidney, enzyme estimations are of more limited value and are of less importance than other techniques such as radiography, cerebrospinal fluid examination and urine analysis. No satisfactory non-invasive antemortem laboratory tests are at present available to assess the severity of damage in respiratory disease of large domestic animals or in gastrointestinal tract disease. The existence of such tests would be of value in sheep, particularly in chronic pneumonia and subclinical gastrointestinal parasitism, as a means of determining the cause of unthriftiness in growing lambs where clinical signs and other laboratory tests may be inconclusive.

CHAPTER 8

ISOENZYME ESTIMATIONS IN RESPIRATORY DISEASE

INTRODUCTION

The development of a biochemical test for detecting the presence and extent of lesions in the lower respiratory tract in man or the domestic animals has not been forthcoming. Considering the diagnostic value of total enzyme and isoenzyme estimations in the detection of damage to other tissues such as liver, heart and muscle, there are relatively few reports of such measurements in diseases of the lung.

In man, Cohen (1970) considered total serum amylase, carbonic anhydrase, creatine kinase, aspartate and alanine aminotransferases, lactate dehydrogenase, and lipase to be useful in the diagnosis of lung diseases. An increase in LDH_3 was observed in lung cancer and increases in the LDH_1:LDH_2 and LDH_5:LDH_4 ratios were found in pulmonary embolism. In the latter, the change in serum isoenzyme pattern was attributed to secondary myocardial and hepatic damage in addition to possible lung damage. Cohen also considered creatine kinase isoenzymes to be of potential diagnostic value. Sax *et al*. (1979) studied "atypical" creatine kinase activity in the serum of patients with pulmonary disease including pulmonary embolism, chronic pulmonary disease and pneumonia. Of 62 patients showing the "atypical" band, which

usually migrates between the MM and MB isoenzymes on electrophoresis, 27% had pulmonary disease, but 77% had chronic heart disease. The presence of this band, which is immunologically distinct but of unknown origin, was therefore not specific for pulmonary disease, and its appearance in the serum was not associated with low arterial partial pressure of oxygen (PO_2) as had been previously suggested. Goffman, Cantrell and Schein (1981) described an increase in total creatine kinase in a patient with a primary carcinoma of the lung. The raised total enzyme level was due to marked increases in the MB and BB isoenzymes, particularly the latter, and the authors recommended the measurement of MB activity as a tumour marker in lung cancer. Gazdar et al., (1981) also noted increased serum BB levels in approximately 1/3 of patients with extensive squamous cell carcinoma of the lung. Increased serum BB levels in lung cancer are not unexpected since neoplastic tissues tend to show a "foetal" isoenzyme pattern which is rich in this isoenzyme and the BB isoenzyme constitutes approximately 73% of the creatine kinase in normal human lung (Tsung, 1976).

In the domestic animals, there appear to be only two reports on the use of enzyme estimations in respiratory disease. Georgiev and Monov (1976) investigated lactate dehydrogenase levels in the serum

and skeletal muscle of pigs with bronchopneumonia. The total serum enzyme level rose from the normal value of 690IU/l to 1380IU/l in affected pigs while in muscle the lactate dehydrogenase activity was more than twice that in normal pig muscle. In the serum, a differential increase in the more anodic isoenzymes was observed but they were not quantified. The authors considered that the changes were characteristic of pigs with bronchopneumonia and were valuable for diagnostic purposes. Ottonello, Ubaldi and Corbella (1979) measured serum aspartate and alanine amino-transferases and lactate dehydrogenase in five horses with acute pulmonary emphysema but only the latter enzyme showed abnormal values. In affected horses there was an increase in the percentage of LDH_4 in serum with a reciprocal decrease in percentage LDH_3 and they considered this change to be of diagnostic value. In sheep, serum enzymes have not been investigated in respiratory disease.

The work described in Chapter 6, Part 2 indicated that unique tissue isoenzyme patterns were obtainable using a combination of lactate dehydrogenase and creatine kinase isoenzyme estimations. In order to determine whether lung-specific patterns could be detected in the serum of sheep in diseases of the lower respiratory tract, these isoenzymes were measured in experimentally induced chronic and acute pneumonia. The work was carried out in collaboration

with the Microbiology Department, Moredun Research Institute, Edinburgh.

All tables in Chapter 8 are presented in Appendix 5.

PART 1 - CHRONIC PNEUMONIA

Proliferative exudative pneumonia is an experimentally reproduced form of naturally occurring atypical pneumonia of lambs. It may be produced by endobronchial inoculation with *Mycoplasma ovipneumonia* with or without *Pasteurella haemolytica* and with or without *M. arginini* (Jones, Gilmour and Rae, 1978), and is characterised by grey or red, well demarcated areas of consolidation mainly affecting the cardiac and apical lobes of the lung. In the experiment to be described, *M. ovipneumoniae* and *P. haemolytica* type A_2 were used to induce the disease. In order to investigate the possibility that any changes in serum isoenzymes might be due to hypoxic damage to the heart and liver, isoenzyme estimations were carried out on tissue homogenates and blood gas analysis was undertaken.

EXPERIMENTAL DESIGN

Twenty, 14 week old, clinically normal Cheviot lambs were divided into two groups each of 10 lambs, a control group (group N) which received no treatment,

and an infected group (group PN). Groups were housed separately throughout the experiment, and were fed pellets containing dried molassed beet pulp and dried grass with added mineral supplement and salt. Lambs from both groups were slaughtered when they reached 45-50kg while the remaining animals were slaughtered 15 weeks after the start of the experiment.

PARAMETERS INVESTIGATED

Serum samples were obtained at weekly intervals from both groups from week 8 to 15 after inoculation of group PN, for estimation of total lactate dehydrogenase and creatine kinase and their isoenzymes. During week 15, arterial blood samples were collected for measurement of the partial pressure of CO_2 and O_2 (PCO_2 and PO_2), bicarbonate, total CO_2 and "base excess". At slaughter, samples of heart, liver and lung were collected from both groups for estimation of total lactate dehydrogenase and its isoenzymes. In the infected group, samples were taken from macroscopically normal and from consolidated areas of lung. Pneumonic lungs were given a "lung lesion score" (the mean percentage of the dorsal and ventral surfaces of the lung showing consolidation) by staff of the Moredun Research Institute. Histological examination of lung lesions was also undertaken by the Moredun Research Institute staff.

In the experiment described in Chapter 6, Part 3, serum creatine kinase isoenzyme estimations in serum were not undertaken as at that time, a means of preventing loss of activity on storage had not been developed. The present experiment therefore provided an opportunity for establishing the normal serum creatine kinase isoenzyme levels in lambs.

MATERIALS AND METHODS

Lambs in group PN were inoculated with a homogenate of pneumonic lung lesions from naturally occurring cases of atypical pneumonia which was prepared as described by Jones, Gilmour and Rae (1978). The homogenate contained *M. ovipneumoniae* and *P. haemolytica* type A_2. 6ml was inoculated intra-tracheally and 7 days later 1ml of an 18 hour broth culture of *P. haemolytica* type A_2 was inoculated intranasally.

Venous blood samples were collected and the serum harvested as previously described. Two 100 µl aliquots of each sample were placed in 0.5ml microcentrifuge tubes for lactate dehydrogenase and creatine kinase isoenzyme studies and the remaining serum in a 75 x 12 mm capped polypropylene tube for total enzyme measurements.

Arterial blood samples were collected from the carotid artery using 21 gauge, 1½" needles (Becton-Dickinson Ltd.) into 10 ml glass syringes (Rocket of

London) containing 0.5 ml (2500 units) Heparin sodium injection BP (Mucous) (Evans Medical Ltd., Greenford, Middlesex) and were kept on ice until analysed, within 2 hours of collection. Analysis was carried out using a Corning 168 pH Blood Gas System (Corning Ltd., Halstead, Essex) which directly measures pH, PCO_2 and PO_2 and calculates bicarbonate (HCO_3^-), total carbon dioxide (TCO_2) and base excess (B.E.).

At slaughter, approximately 5g samples were obtained from the apex of the heart and the caudate lobe of the liver. Samples of normal areas of lung were taken from the posterior border of the diaphragmatic lobe and samples of consolidated lung from the cardiac lobe. The lambs were killed by stunning with a captive bolt pistol followed by exsanguination. Tissues were collected immediately after death and kept on ice until homogenates were made, as described on page 180. within a few hours of collection. One 100µl aliquot of each homogenate was placed in a separate 0.5 ml microcentrifuge tube for lactate dehydrogenase iso-enzyme electrophoresis while the remainder was placed in a 75 x 12 mm polypropylene tube for total lactate dehydrogenase estimation. Creatine kinase estimations were not undertaken in the tissues because when this experiment was carried out, a method for preventing loss of creatine kinase activity in tissues (but not sera) on storage had not been developed (see Chapter

6, Part 1) and erroneous results would therefore have been produced. Sera and homogenates were stored at -70°C until analysed. Total creatine kinase and creatine kinase isoenzymes in serum and total lactate dehydrogenase and lactate dehydrogenase isoenzyme estimations in serum and tissues were carried out as previously described.

Lung lesion scores were measured by drawing the lesions on lung diagrams for both dorsal and ventral surfaces and estimating the percentage of the lungs showing consolidation by a point-grid system. The score was a mean of the dorsal and ventral measurements. Examples of lung diagrams similar to those obtained in this experiment are shown in Part 2 of this Chapter.

RESULTS

The mean values and standard deviations for total lactate dehydrogenase and creatine kinase and their isoenzymes in serum were calculated. The mean values for each total enzyme and isoenzyme, the latter being expressed both as an absolute value in IU/l and as a percentage were compared between groups at each sampling by Student's t-test. The results are shown in Tables 8.1 - 8.5.

The results shown in Table 8.3 indicate that the total lactate dehydrogenase did not differ

significantly between groups from week 8 to week 15. Lactate dehydrogenase isoenzymes showed minor differences in their serum levels between groups, which just reached statistical significance ($p < 0.05$) but no trend was apparent. Total creatine kinase and its isoenzymes showed no differences between groups (results of Student's t-test not shown). The MM_2, MM_1, MB_1 and BB isoenzymes but not the MB_2 isoenzyme were present in the serum.

Mean values and standard deviations were calculated for lactate dehydrogenase and its isoenzymes in liver, heart and lung samples from groups N and PN (Tables 8.6 and 8.7). One sheep in group N showed consolidated lung lesions. Student's t-test was carried out to compare the values between the two groups (Table 8.8) and between normal lung from group N and consolidated lung from group PN (Table 8.9).

From Table 8.8, only minor differences were observed in tissue lactate dehydrogenase isoenzyme levels, while the total enzyme levels did not differ significantly between groups. Liver from group N had a higher $\%LDH_2$ and a lower $\%LDH_1$ than from group PN and normal areas of lung had a higher $\%LDH_1$ in group N than group PN, but these differences only just reached statistical significance ($p < 0.05$). However, marked differences were observed between normal and consolidated lung tissue.

Total lactate dehydrogenase was higher in abnormal than normal lung with a higher absolute level of LDH_2, LDH_3, LDH_4 and LDH_5 but no significant difference in the LDH_1 level. The isoenzyme distribution also differed, with a higher percentage of LDH_4 and LDH_5 in abnormal lung and a reciprocal decrease in the percentage of LDH_1 and LDH_3. Thus, although the absolute LDH_3 level was higher in abnormal lung, the very large increase in the percentage of LDH_4 and LDH_5 resulted in a relative decrease in the percentage of LDH_3.

Blood gas analysis of arterial samples from groups N and PN are shown in Table 8.10. The differences between groups were not statistically significant for any of the parameters measured (results of Student's t-test not shown).

During the period when serum samples were collected, infected lambs showed increased respiratory rates, coughing and ocular and nasal discharges. At post-mortem examination, typical red/grey consolidation of the anteroventral parts of the lungs, and in some cases pleurisy, were evident. Control lambs were clinically normal throughout the experiment although one showed small anteroventral consolidated areas at post-mortem examination and others showed very small patches of consolidation. Mean lung lesion score in group N was 1.6% ($s \pm 1.94$; range 0 - 6.5%) and in

group PN 19.9% (s $\pm$ 10.85 ; range 4.4 - 35.4%).

Histopathology of consolidated areas of lung, which was carried out by the Moredun Research Institute, revealed characteristic changes of proliferative exudative pneumonia with bronchiolar hyperplasia, hyaline scars, perivascular fibrosis, lymphoid hyperplasia and accumulation of macrophages, giant cells and polymorphs.

DISCUSSION

In pulmonary disease in man, Cohen (1970) has stated that it cannot be said with certainty that the serum enzymes which are measured originate from the damaged lung itself. This statement still appears to be valid. The serum lactate dehydrogenase isoenzyme changes described by Cohen (1970) and the creatine kinase isoenzyme study by Sax et al. (1979), which have been discussed more fully in the introduction to this chapter, indicate that in human pulmonary disease, serum isoenzyme changes

(a) may be due to secondary damage to organs other than the lung

and (b) are not specific to pulmonary disease.

In pigs with bronchopneumonia, Georgiev and Monov (1976) observed a marked increase in total serum lactate dehydrogenase which was associated with an

increase in the more anodic isoenzymes, but since the lactate dehydrogenase isoenzyme distribution in porcine lung does not seem to have been reported, an explanation for their findings cannot be given. An increase in the LDH_4: LDH_3 ratio in the serum of horses with pulmonary emphysema (Ottonello, Ubaldi and Corbella, 1979) was considered to be due to release from damaged lung tissue, but in fact LDH_3 and LDH_4 constitute approximately equal proportions of the lactate dehydrogenase activity in equine lung (31.7% and 29.4%, respectively), with lower proportions of the other three isoenzymes (Thornton and Lohni, 1979).

Cohen (1970) suggested that an increase in the LDH_1:LDH_2 ratio and in the LDH_5:LDH_4 ratio in pulmonary embolism in man were due to secondary damage to other organs such as heart, haemolysed red blood cells within the lung or an engorged renal cortex (increased LDH_1) or hepatic injury due to venous hypertension and central venous hepatic necrosis (increased LDH_5). In the present study, the possibility that impaired gaseous exchange in proliferative exudative pneumonia might result in secondary hypoxic damage to other organs was investigated by blood gas analysis and lactate dehydrogenase isoenzyme estimations in myocardium and liver. No significant differences were observed in pH, PO_2, PCO_2, HCO_3^-, base excess or total CO_2 in arterial blood between infected and control animals (Table

8.10), but had the samples been collected after exercise rather than at rest, the likelihood of detecting differences between groups would have been greater. The PO_2 and PCO_2 values are in agreement with those obtained by Mitchell and Williams (1975) in 17-20 week-old lambs, but the pH (7.312 and 7.314 for groups N and PN respectively) falls below their mean normal value of 7.45. The apparently low pH cannot be due to a respiratory acidosis since it was observed in group N as well as PN, and it was not associated with an increased PCO_2.

Changes in the isoenzyme complement of tissues in hypoxic states have been described, for example in rabbits, the effect on the enzymes of the aortic wall of mechanical injury to the aorta in the presence of concurrent chronic hypoxia was studied by Lindy et al. (1974). Total lactate dehydrogenase increased with an elevation of the more cathodal isoenzymes. This increase in the isoenzymes with a predominance of M subunits was considered to be an adaptation to hypoxia since M subunits facilitate the formation of lactate from pyruvate and therefore the formation of the NAD^+ necessary for the maintenance of glycolysis. In this experiment little effect on the lactate dehydrogenase isoenzyme distribution in liver was evident with an increase in % LDH_1 and a decrease in % LDH_2 in infected animals which just reached statistical significance, while no

differences were observed in the isoenzymes in myocardium. Chronic pneumonia did not result in a detectable decrease in the arterial PO_2 on the one occasion on which blood gas analysis was undertaken and this may account for the fact that only a few changes, which just reached statistical significance occurred in the isoenzyme distribution in two of the organs whose function was likely to be impaired by hypoxia.

The comparison between total lactate dehydrogenase and its isoenzymes in normal areas of lung from group N and consolidated areas of lung from group PN revealed marked differences which would be of great importance in the interpretation of abnormal serum lactate dehydrogenase levels in pneumonia of this type. The total enzyme level in IU/g protein was a mean of 4.4 times higher in consolidated lung and was associated with a very substantial absolute increase in LDH_2, LDH_3, LDH_4 and LDH_5, while the percentage distribution showed an increase in the percentage of LDH_4 and LDH_5 and a resultant reciprocal decrease in the percentage of LDH_1 and LDH_3. Thus in chronic pneumonia, any changes in serum lactate dehydrogenase isoenzymes may reflect the isoenzyme distribution in the lesions rather than in the normal areas of lung. This is particularly likely since the mean absolute activities of LDH_2, LDH_3, LDH_4 and LDH_5 were very high compared to the values in normal lung (mean values of 3.9, 2.5, 9.0 and 29.2 times higher, respectively). The lung

lesions therefore had a considerable potential for altering the serum level of these four isoenzymes, but in particular LDH_5 which represents the least proportion of the lactate dehydrogenase activity in normal lung and the greatest proportion in consolidated lung. The mean absolute level of LDH_5 in the lung lesions was actually higher than the mean total lactate dehydrogenase level in normal lung tissue. The marked differences in the isoenzyme levels in normal and abnormal lung are likely to be due to changes in the predominant cell types present as a result of lymphoid hyperplasia and infiltration of macrophages, giant cells and polymorphs but the isoenzyme distribution in these cells has not been reported.

In this experiment, no changes occurred in the serum isoenzyme levels associated with release from either the normal or the abnormal areas of lung, even in the presence of extensive consolidated lesions, and total lactate dehydrogenase, total creatine kinase and creatine kinase isoenzymes in the serum did not differ significantly between infected and control lambs and only minor changes were observed in the lactate dehydrogenase isoenzyme levels during the chronic phase of proliferative exudative pneumonia. The failure to detect increases in the cathodic

isoenzymes whose activity increased markedly in consolidated lung tissue may be associated with one or more of the following factors:

(a) the more cathodic isoenzymes have a shorter half-life in the blood than LDH_1, for example the second exponential phase in the removal or inactivation of LDH_1 in the plasma is 48 hours compared with 8 hours for LDH_5 (Boyd, 1967).

(b) the diluting effect of the serum may be such that increases in total enzyme and isoenzyme levels are not detectable after release from damaged tissue.

(c) enzymes may not be released from chronically damaged lung tissue.

The results of this investigation indicate that consolidated lung lesions of chronic proliferative exudative pneumonia have markedly different lactate dehydrogenase isoenzyme levels to normal lung, a fact which would be of considerable importance in the interpretation of serum isoenzyme levels in this disease. However, in the presence of characteristic clinical signs, post-mortem and histological findings of the disease there was no effect on blood gases, and no clinically significant changes occurred in the total or isoenzyme levels of lactate dehydrogenase or creatine kinase in the serum. Such measurements are therefore unlikely to be of value in the diagnosis

of atypical pneumonia, the naturally occurring form of the disease.

In addition to the investigation into serum isoenzyme levels in chronic pneumonia, the normal creatine kinase isoenzyme levels in Cheviot lambs was established since they have not been previously reported in sheep. The mean values of 49 IU/l at 25°C (approximately 74 IU/l at 30°C) obtained by Boss, Gerber and Tschudi (1979) and the value of 21 IU/l at 25°C (approximately 32 IU/l at 30°C) in adult sheep (Boehringer, 1979) correlate well with the mean total serum creatine kinase levels obtained in this experiment (see Table 8.4).

Four isoenzymes were present in serum which corresponded in migration distance to MM_2, MM_1, MB_1, and BB in ovine tissues (see Chapter 6, Part 2). Reports on serum creatine kinase isoenzyme levels in other species, which have been described in detail in Chapter 5, indicate that only the MM and traces of the MB isoenzyme are normally present in human serum, substantial proportions of MM and MB are found in equine serum and MM and BB in porcine serum.

The presence of a cathodally migrating band of creatine kinase has been reported in the serum of some patients after myocardial infarction and was considered to be of mitochondrial origin (Bark, 1980) but Kwong and Arvan (1981) state that a cathodic

variant could represent either mitochondrial creatine kinase or adenylate kinase, which is known to interfere with creatine kinase isoenzyme estimations. Bohner et al. (1982) described an isoenzyme which he called macro-CK type 2 in the serum of patients with severe illness from a variety of causes, which was considered to be of mitochondrial origin. This view was substantiated by Stein et al. (1982), who demonstrated the similarity between serum macro-CK type 2 and mitochondrial creatine kinase isolated from human tissues. The presence of this band does not appear to have been described in normal human serum. The existence of anodally migrating creatine kinase bands on electrophoresis in addition to the MM, MB and BB isoenzymes in human serum have been described between MM and MB (Lim, 1975; Ljungdahl and Gerhardt, 1978; Sax et al. 1979; Urdal and Landaas, 1979; Chapelle and Heusghem, 1980; Bohner et al., 1982). The formation of these bands has been attributed to polymeric aggregation of creatine kinase monomers, or to complexing of creatine kinase isoenzymes with immunoglobulins, lipids, lipoproteins or other unknown components of serum (Kwong and Arvan, 1981). One of these bands, named macro-CK type 1 is found mainly in elderly women with a variety of disorders and is known to consist of the BB isoenzyme bound to IgG or IgA (Lang and Würzburg, 1982). Less frequently,

bands have been described between MB and BB (Lim, 1975; Sax et al., 1979). Additional anodally migrating bands, like the cathodally migrating band, have not been described in normal serum. Recently, a five band pattern almost identical to that of normal ovine serum has been reported in a patient with severe heart disease (Csako et al., 1982).

The preceeding discussion outlines some of the circumstances under which additional creatine kinase bands appear in human serum and some explanations for their existence. Consideration of these reports in relation to the present findings in lamb serum allowed the following conclusions to be made:

(a) the cathodally migrating isoenzyme in ovine serum (MM_2) does not represent adenylate kinase activity since the staining procedure was known to be specific for creatine kinase

(b) the cathodally migrating isoenzyme is likely to be the mitochondrial isoenzyme in view of its position on the electrophoresis gel and because it was also detected in tissue homogenates

(c) MB_1 is unlikely to be formed by binding of one of the other isoenzymes to serum constituents such as immunoglobulins or lipoproteins since it was also detected in tissues

Although the nature of the additional bands in normal ovine serum compared with normal serum of other species is uncertain, this study has served to

demonstrate their existence and to provide figures for the normal levels in lambs serum.

PART 2 - ACUTE PNEUMONIA

Type A serotypes of *Pasteurella haemolytica* cause enzootic pneumonia in all ages of sheep and may result in generalised septicaemia and sudden deaths in lambs under 2 months old.

The experimental reproduction of acute pasteurella pneumonia is most reliably achieved

(a) in hysterectomy-derived specific pathogen-free (SPF) lambs.

(b) in the presence of intercurrent disease.

In the experiments to be described, two different intercurrent diseases (tick-borne fever and parainfluenza virus type 3) were induced prior to inoculation with *P. haemolytica* type A_2.

A. PASTEURELLA HAEMOLYTICA TYPE A_2 AND TICK-BORNE FEVER

EXPERIMENTAL DESIGN

Eight, 4 week old, crossbred SPF lambs were inoculated with *Cytoecetes phagocytophila*, the causal agent of tick-borne fever (TBF) and 7 days later with an aerosol of *P. haemolytica* type A_2. Lambs were

housed in one pen throughout the experiment and were fed on reconstituted Carnation milk. Lambs died or were killed if very severely affected during the experiment, and survivors were killed 7 days after infection with P. haemolytica.

PARAMETERS INVESTIGATED

Venous blood samples were collected on the day before inoculation with P. haemolytica and 1, 3 and 7 days after inoculation, for estimation of total lactate dehydrogenase and creatine kinase and their isoenzymes. Lambs were examined for clinical signs of respiratory disease for the first 5 days after inoculation. One point was assigned for each of four parameters - pyrexia (rectal temperature $> 40.6^{\circ}C$), dullness, abnormal respiration (tachypnoea or abdominal respiration) and death. After death, the lamb was assigned a score of 4 for each subsequent day. Clinical scores for each day were added to give a total clinical score. At post-mortem examination, a lung score was given according to the extent of the lesions. The absence of lesions scored 0, 5-10% of the lung with lesions scored 5, 11-25% scored 10 and more than 25% scored 20. Clinical and lung lesion scores were estimated and histological examination of lung lesions performed by staff of the Moredun Research Institute.

MATERIALS AND METHODS

Lambs were inoculated with a stabilate of Cytoecetes phagocytophila of ovine origin, known to provoke a mild febrile reaction in sheep.

P. haemolytica was administered intranasally and intratracheally by inhalation of an aerosol of organisms in phosphate-buffered saline (Sharp et al., 1978). The aerosol contained $10^{6.05}$ organisms/litre and was produced by means of a Wright's nebuliser (Aerosol Products (Colchester) Ltd., Essex).

Venous blood samples were collected for harvesting of serum as previously described. Two 100µl aliquots of each sample were placed in 0.5ml microcentrifuge tubes for isoenzyme studies and the remaining serum in a 75 x 12mm polypropylene tube for total enzyme estimations. Sera were stored at -70°C until analysed. Total lactate dehydrogenase, total creatine kinase and their isoenzymes were estimated as previously described.

Lambs were killed by the intravenous injection of 10ml Pentobarbitone Sodium BP (Vet) 20% w/v (Euthatal(R), May and Baker Ltd., Dagenham, Essex) followed by exsanguination.

RESULTS

The mean values and standard deviations were calculated for the total serum enzyme and isoenzyme

levels and the results are presented in Tables 8.11 - 8.14. In order to determine whether the values at 1 and 3 days post-inoculation changed with respect to the pre-inoculation level, paired t-tests were carried out on the individual results for the five lambs sampled on day 3 after inoculation with P. haemolytica. Only two lambs survived until day 7 which was an insufficient number for comparison with the pre-infection values. The results of the paired t-tests for lactate dehydrogenase are shown in Table 8.15.

From Table 8.15, the absolute level of LDH_2 was significantly higher at 1 day post-inoculation and LDH_3 at 1 and 3 days post-inoculation than on the day before inoculation, but these changes were not highly significant and were not reflected in changes in the total lactate dehydrogenase level. The percentage of LDH_3 however, was higher at 1 and 3 days post-inoculation than at 1 day pre-inoculation, the difference being highly significant at day 1 ($p<0.001$).

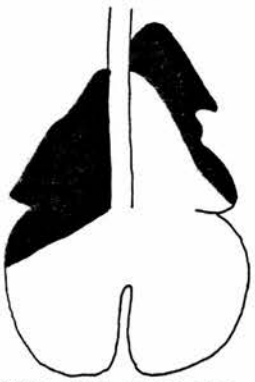
Total serum creatine kinase and its isoenzymes did not show any significant change at days 1 and 3 with respect to the pre-inoculation level (results of paired t-tests not shown).

During the first five days after inoculation with P. haemolytica, all lambs showed dullness, temperatures exceeding 40.6°C, increased respiratory rates, abdominal

respiration or coughing. The mean total clinical score was 11 (s $\pm$ 6.2; range 0 - 18). Post-mortem examination of lambs dying in the first 3 days after inoculation with P. haemolytica showed deep red, firm, oedematous haemorrhagic lesions with fairly distinct borders affecting the ventral parts of the cranial, intermediate and caudal lobes. A clear yellow gelatinous material was adherent to the lesions in some lambs. In those surviving until days 4 to 7, the lesions extended up to the dorsal border of the lung and had a dark red haemorrhagic centre which was sometimes encapsulated and surrounded by pale grey areas of consolidation. The mean lung lesion score was 15(s $\pm$ 7.6; range 0-20). Examples of lung lesion diagrams are shown in Fig. 8.1. Histopathology, which was carried by the staff of the Moredun Research Institute, showed the presence of pleomorphic cells with intensely basophilic nuclei surrounding necrotic foci and the alveolar capillaries. This layer of cells was surrounded by a connective tissue capsule followed by a zone of consolidated alveoli infiltrated by macrophage-like cells. The bronchiolar epithelium was hyperplastic or had sloughed in the consolidated areas.

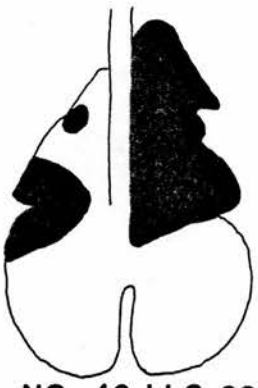
To determine whether a significant correlation existed between clinical and lung lesion scores and

Figure 8.1. Typical lung lesion diagrams from lambs with acute pasteurella pneumonia (Chapter 8, Part 2, Experiment A). Only the diagrams for the dorsal aspect of the lungs are shown. Black areas indicate lesions. LLS = lung lesion score.

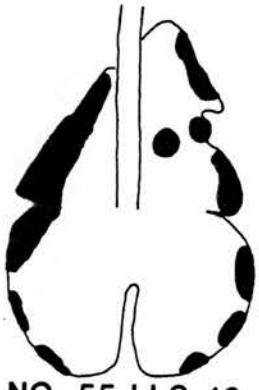


NO. 25 LLS 20

NO. 36 LLS 20



NO. 46 LLS 20



NO. 55 LLS 10

NO. 71 LLS 20

LUNG LESION DIAGRAMS–DORSAL ASPECT

total enzyme and isoenzyme levels, linear regression analysis was carried out on the total serum enzyme and isoenzyme values at the last sampling before death for each of the eight lambs. There was no significant correlation between the total serum levels of either enzyme nor their isoenzymes (absolute or percentage levels) and either clinical or lung lesion score (results not shown).

B. PASTEURELLA HAEMOLYTICA TYPE A_2 AND PARAINFLUENZA VIRUS TYPE 3

EXPERIMENTAL DESIGN

Five, 9 week old, crossbred SPF lambs were infected with parainfluenza virus type 3 (PI3) 7 days before inoculation with *P. haemolytica* type A_2 by aerosol. Lambs were penned with others which had been inoculated with PI3 virus only, and were fed on sterilised hay and proprietary lamb weaner pellets. As in the previous experiment, lambs died or were killed if showing severe clinical signs and surviving lambs were killed 7 days after inoculation with *P. haemolytica*.

PARAMETERS INVESTIGATED

Venous blood samples were collected immediately prior to inoculation with *P. haemolytica* and daily

for the next four days for estimation of total lactate dehydrogenase and creatine kinase and their isoenzymes. In addition, samples of normal and abnormal areas of lung from two lambs which died two days after inoculation with P. haemolytica were collected for measurement of the same parameters as in serum. Unfortunately, due to the timing of the deaths, it was not possible to collect fresh material from other lambs. Clinical and lung lesion scores were calculated as indicated in the previous experiment. Clinical scores were recorded for the first 5 days after inoculation. Histological examination was again carried out by staff of the Moredun Research Institute.

MATERIALS AND METHODS

Inoculation of lambs with PI3 virus was carried out by intra-tracheal and intra-nasal routes. Virus for inoculation had a $TCID_{50}$ of 10^7 per ml (i.e. a titre of 10^7 per ml was sufficient to infect 50% of cells in a tissue culture). P. haemolytica was administered by aerosol, as in the previous experiment, the aerosol containing approximately $10^{4.3}$ organisms/ litre.

Collection of venous blood samples, preparation of lung samples and total enzyme and isoenzyme estimations were carried out as previously described

and division into aliquots was as described in the preceeding experiment.

Lambs were killed by intravenous injection of 10ml Euthatal$^{(R)}$ (May and Baker Ltd.) followed by exsanguination.

RESULTS

Means and standard deviations were calculated for the total serum enzyme and isoenzyme levels and the results are shown in Tables 8.16 - 8.19. Paired t-tests were carried out to compare the results for the four animals which were sampled immediately before inoculation with P. haemolytica (day 0) with the results for the same animals one day after inoculation. Paired t-tests could not be carried out on the data for subsequent days due to an insufficient number of the lambs sampled on day 0 remaining.

The results of the paired t-tests indicated that total serum lactate dehydrogenase was significantly higher ($t = -6.371$; $p < 0.01$) and the absolute level of creatine kinase MB_1 significantly lower ($t=3.314$; $p < 0.05$) at day 1 post-inoculation than immediately prior to inoculation with P. haemolytica. Differences between total creatine kinase, lactate dehydrogenase isoenzymes and the MM_2, MM_1 and BB isoenzymes of creatine kinase were not significant (results of paired t-tests not shown).

The mean values of total lactate dehydrogenase and creatine kinase and their isoenzymes (percentage levels and absolute levels in IU/g protein) in normal lung and lung lesions are shown in Tables 8.20 and 8.21.

The mean total lactate dehydrogenase level was higher in the lesions than in macroscopically normal areas of lung as a result of higher absolute LDH_4 and LDH_5 levels. This trend was also reflected in the percentage isoenzyme levels with abnormal lung showing a higher mean percentage of LDH_4 and LDH_5 and lower percentages of LDH_1 and LDH_2. The mean values for total creatine kinase and its isoenzymes however, were similar in normal and abnormal lung. Unfortunately, it was not possible to compare the values using statistical methods in view of the small number of lambs from which lung samples were collected.

The clinical signs, post-mortem findings and histopathology were as described in the previous experiment. Mean total clinical score was 12(s $\pm$ 6.5; range 3-18) and mean lung lesion score was 15 (s $\pm$ 7.1; range 5-20).

Linear regression analysis was carried out to determine whether total serum enzyme and isoenzyme levels at the sampling prior to death in all five lambs showed a significant correlation with total

clinical score or with lung lesion score. Neither total enzyme levels nor isoenzyme levels expressed either in IU/l or as a percentage showed a significant correlation with clinical or lung lesion scores.

DISCUSSION (EXPERIMENTS A AND B)

The experimental reproduction of acute pasteurella pneumonia in lambs can now be reliably achieved by intra-nasal and intra-tracheal inoculation with an aerosol of P. haemolytica in SPF lambs, in the presence of mild intercurrent disease. In experiment A, the inoculation of lambs with Cytoecetes phagocytophila produced a mild pyrexia while in experiment B, inoculation with PI3 virus produced dullness, anorexia, pyrexia and hyperpnoea from days 4 to 6 after inoculation but by day 7, the clinical signs had largely abated. In spite of the fact that the intercurrent disease in experiment B affected the respiratory tract whereas in experiment A it did not, the clinical signs, post-mortem and histological findings were identical after subsequent inoculation with P. haemolytica (Gilmour, 1982). In both cases a severe respiratory disease was produced within 24 hours of exposure to P. haemolytica, six of the eight lambs in experiment A and three of the five lambs in experiment B dying or being killed in extremis within 7 days of inoculation with the bacterium.

Although the acute pneumonia produced in the presence of the two different intercurrent diseases was apparently identical, the effect on total enzyme and isoenzyme levels in the serum differed in the two experiments. In experiment A, a significant increase in both the absolute and percentage levels of LDH_3 occurred at days 1 and 3 after inoculation with P. haemolytica with respect to the pre-infection level. When the values were expressed as a percentage, the difference was highly significant. No significant change however, occurred in the total lactate dehydrogenase, total creatine kinase or creatine kinase isoenzyme levels in the serum.

When the data for individual lambs was examined, LDH_1 was found to be the predominant isoenzyme in all eight lambs before inoculation with P. haemolytica, but by day 1 after inoculation, LDH_3 had become the predominant isoenzyme in two lambs, and by day 3 in a further two lambs. There was no correlation between this increase in absolute and percentage levels of LDH_3 and either clinical or lung lesion score. The change in the serum lactate dehydrogenase isoenzyme profile was therefore an indicator of the presence but not the degree of severity of acute pneumonia, and clearly the absence of such a change does not indicate the absence of either clinical signs

or lung lesions. An increase in the serum LDH_3 level is consistent with leakage of lactate dehydrogenase from lung tissue since it is the predominant isoenzyme in normal ovine lung and in lesions from the lungs of lambs with acute pasteurella pneumonia (see Table 6.9 and Table 8.20).

In experiment B, the number of lambs was insufficient to allow comparison between the levels after with those before inoculation with P. haemolytica, except at day 1 after inoculation. At day 1, the total serum lactate dehydrogenase level was significantly higher ($p < 0.01$) than before inoculation and the absolute creatine kinase MB_1 level was lower, but the latter only just reached statistical significance and was considered to be of little practical importance. The mean absolute and percentage values of LDH_1, LDH_2 and LDH_3 were higher and LDH_4 and LDH_5 lower at day 1 than before inoculation, but these changes were not statistically significant and no change in the serum lactate dehydrogenase isoenzyme distribution occurred, with LDH_1 predominating over LDH_3 both before and after infection. The data for the total creatine kinase activity in Table 8.18 indicate that the mean level was markedly higher at days 0 and 1 than at subsequent samplings. This was mainly due to a higher MM_1 level and was considered to be due to release from skeletal muscle in which it constitutes 100% of the total activity, as

the lambs were subjected to minor trauma during transport to the place where the aerosol was administered, and during administration of the aerosol. By day 2, the total enzyme level had decreased due to its serum half-life of only 62 minutes (Boyd, 1976).

As in chronic proliferative exudative pneumonia, lung lesions showed a higher total lactate dehydrogenase level and higher absolute and percentage levels of LDH_4 and LDH_5 than normal areas of lung in acute pasteurella pneumonia, but in the latter, the isoenzyme distribution did not change to the extent that LDH_5 replaced LDH_3 as the predominant isoenzyme. Thus in experiments A and B, any lactate dehydrogenase released from either normal or abnormal areas of lung would be expected to result in an increase in serum LDH_3 in particular, but only in experiment A was this observed. The change in lung isoenzyme distribution is likely to be due to a change in the predominant cell types, the presence of pleomorphic cells and infiltration by macrophages being a feature of pasteurella pneumonia. Creatine kinase BB predominated in normal and abnormal areas of lung in acute pasteurellosis and would be expected to increase in the serum if released from damaged lung, but the serum level of this isoenzyme did not change in either experiment.

The reasons for the differences in the effect on serum enzyme and isoenzyme levels of acute pasteurella

pneumonia associated with two different predisposing diseases is uncertain, particularly since the mean clinical and lung lesion scores were not significantly different in the two experiments and the post-mortem and histological findings were similar, and in the apparent absence of previous reports on the effect of acute pneumonia on serum enzyme levels in any species, no comparisons can be made between my results and those of other authors. The fact that only small numbers of lambs were available may contribute to the inconsistencies observed but the severity of the disease is such that lamb numbers are certain to fall during the course of the experiments.

CONCLUSIONS FROM CHAPTER 8

In chronic proliferative exudative pneumonia with a mean value of 20% of the lung affected by consolidation, no diagnostically important changes in the total or isoenzyme levels of lactate dehydrogenase or creatine kinase in the serum occurred. This failure to detect changes may be associated with the apparent absence of hypoxia in affected lambs. A markedly higher total lactate dehydrogenase activity was detected in consolidated lung lesions than in normal lung due to increases in the absolute levels of LDH_2, LDH_3, LDH_4 and LDH_5, and this was considered to be due to changes in the predominant cell types present. The normal

serum creatine kinase isoenzyme levels have been established for the first time in lambs, with four isoenzymes being present, corresponding to MM_2, MM_1, MB_1 and BB in ovine tissues. The total serum enzyme and isoenzyme measurements undertaken are not likely to be of value in the detection of naturally occurring atypical pneumonia.

In acute pasteurella pneumonia in SPF lambs superimposed on tick-borne fever, a significant increase in serum LDH_3 occurred and in four of the eight lambs this resulted in LDH_3 replacing LDH_1 as the predominant isoenzyme. The increase in serum LDH_3 was considered to be due to release from lung tissue. In pasteurella pneumonia superimposed on PI3 infection, a significant increase in total serum lactate dehydrogenase occurred but this was not associated with a significant increase in any particular isoenzyme. Thus, an increase in LDH_3 may confirm the presence of acute pneumonia, but clearly its absence does not indicate the absence of lesions. As in chronic proliferative, exudative pneumonia, lung lesions from cases of acute pasteurella pneumonia showed an increase in the more cathodic isoenzymes, LDH_4 and LDH_5, but this change in the distribution was less marked than in chronic pneumonia and LDH_3 was still the predominant isoenzyme.

CHAPTER 9

ISOENZYME ESTIMATIONS IN GASTROINTESTINAL PARASITISM

INTRODUCTION

Serum enzyme and especially isoenzyme levels have been largely neglected as means of assessing the severity of tissue damage in gastrointestinal disease.

In man, intestinal obstruction may cause elevations in serum amylase and lipase and intestinal strangulation or infarction can produce increased serum transaminase, lactate dehydrogenase and alkaline phosphatase levels (Ticktin and Trujillo, 1970) but increases in the intestinal isoenzyme of the latter are very rarely observed in intestinal disease (Kaplan, 1972). In patients with gastric and colonic neoplasia, particularly those with metastases, increases have been reported in serum glucosephosphate isomerase and γ-glutamyl transpeptidase (Munjal and Brady, 1978; Munjal, 1980). In carcinoma of the colon in man, the presence of three atypical creatine kinase bands has been reported on the cathodal side of MB, at the application point and on the anodal side of MM on electrophoresis (Kanemitsu, Kawanishi and Mizushima, 1981). The atypical bands were shown to be a complex of the MM isoenzyme with IgA and a β-lipoprotein, a macromolecular form due to aggregation of isoenzymes and a complex of the MM isoenzyme with IgG. In patients undergoing gastrointestinal tract surgery, serum creatine kinase MB has been found to increase

in 40% and BB in 13% of cases (Tsung, 1981). Tsung found this result surprising since BB is present at a higher concentration than MB in human intestine.

In dogs, acute experimental colonic infarction produces a rise in total serum lactate dehydrogenase and creatine kinase from approximately 40 to 130 IU/l and from 30 to 1450 IU/l, respectively. Total lactate dehydrogenase reached a peak at 6-9 hours after infarction and was associated with an increase in LDH_3 which together with LDH_2, predominates in canine small and large intestine wall. Total creatine kinase reached a peak at 12 hours with increases in all three isoenzymes but particularly the MM isoenzyme. The MM and MB isoenzymes reached a peak at the same time as the total enzyme level, but the BB isoenzyme was maximal at 6 hours after infarction. A combination of serum lactate dehydrogenase and creatine kinase estimations were considered to be of diagnostic value (Graeber et al., 1981).

In pigs, plasma alkaline phosphatase activity is reported to decrease in infection with Strongyloides ransomi (Enigk, Dey-Hazra and Dimitrov, 1973) while in 81% of horses with intestinal parasitism and 89% of those with diarrhoea, "intestinal" alkaline phosphatase, as estimated by L-phenylalanine inhibition, was said to increase in the serum (Blackmore and Palmer, 1977). Similarly, serum alkaline phosphatase

in cattle with intractable diarrhoea was more sensitive to L-phenylalanine than that of normal cattle (Healy, 1971).

In sheep, enzyme studies in gastrointestinal disease have been mainly confined to the measurement of total enzyme levels in the mucosa with some reports of changes in the levels of digestive enzymes in the serum or plasma. Examples of such studies include a decrease in alkaline phosphatase, lactase, and maltase in the intestinal mucosa of lambs with Nematodirus battus infection (Coop, Mapes and Angus, 1972; Coop, Angus and Mapes, 1973), a decrease in leucine aminopeptidase, alkaline phosphatase and maltase in the anterior third of the small intestine of sheep with Trichostrongylus colubriformis infection (Coop and Angus, 1975), and increased plasma pepsinogen levels during larval emergence from the glands of the abomasum in Ostertagia circumcincta infection (Coop, Sykes and Angus, 1977). The measurement of serum enzymes other than those associated with digestion does not appear to have been described as a means of detecting gastric or intestinal damage in sheep.

In the four experiments to be described, lactate dehydrogenase, creatine kinase and their isoenzymes were measured in the serum in chronic gastrointestinal parasitism, in the acute and chronic phases of

parasitic gastritis caused by two different nematodes and in acute parasitic gastritis. The work was carried out in conjunction with members of the Parasitology and Pathology Departments, Moredun Research Institute, Edinburgh.

All tables from Chapter 9 are presented in Appendix 6.

PART 1 - CHRONIC GASTROINTESTINAL PARASITISM

In this experiment, the effect of chronic infection with Trichostrongylus vitrinus, which inhabits the first 5-7 metres of the small intestine, with or without concurrent infection with the abomasal parasite, Ostertagia circumcincta was investigated.

EXPERIMENTAL DESIGN

Twenty-one, clinically normal 3½ month old Suffolk x Greyface lambs which had been reared under worm-free conditions were divided into 3 groups of 7 animals. Group T received 2000 T. vitrinus larvae daily, 5 days per week, group OT received 1500 O. circumcincta and 2000 T. vitrinus larvae daily, 5 days per week and group C were untreated. Lambs were kept in separate metabolism crates in the same house throughout the experiment and were fed on ruminant A, a complete diet. The composition of this diet has been described by Wainman, Blaxter and Pullar (1970).

After 14 weeks, groups T and OT were slaughtered. Group C lambs were required for another experiment and were therefore not slaughtered.

PARAMETERS INVESTIGATED

Serum samples were obtained at weekly intervals from all three groups between 10 and 13 weeks after the experiment started and measurement of total lactate dehydrogenase, total creatine kinase and their isoenzymes was undertaken.

At slaughter of groups T and OT, samples of abomasum and anterior small intestine were collected for estimation of total lactate dehydrogenase and its isoenzymes and the gastrointestinal tract was examined macroscopically. Creatine kinase was not estimated in tissues as a means of storing tissue samples without loss of activity had not been developed at this stage.

Additional parameters which were investigated by the staff of the Moredun Research Institute included worm counts and faecal egg counts.

MATERIALS AND METHODS

T. vitrinus and O. circumcincta larvae from strains which have been maintained in the Moredun Research Institute for several years by serial passage through sheep were administered orally as an aqueous suspension.

Venous blood samples were collected and the serum harvested and stored as previously described and aliquots taken as in Chapter 8. Total lactate dehydrogenase and creatine kinase and their isoenzymes were estimated as previously described.

Lambs in groups T and OT were killed by injection of 10ml Euthatal$^{(R)}$ followed by exsanguination. Immediately after death, samples of mucosa were taken from the fundus of the abomasum and from the small intestine approximately 20cm from the pylorus and were kept on ice until homogenates were made, within 10 hours of collection. Homogenates were prepared as described in Chapter 6, Part 2.

RESULTS

Means and standard deviations were calculated for total serum lactate dehydrogenase and creatine kinase and their isoenzymes and the results are presented in Tables 9.1 - 9.4. Paired t-tests were carried out to compare the mean values of these parameters at each sampling. The results are shown in Tables 9.5 and 9.6.

Minor differences in total serum lactate dehydrogenase and LDH_1, LDH_2, LDH_4 and LDH_5 occurred (see Table 9.5), but these were not consistently observed at each sampling and no clear trend was evident. LDH_3 however, when expressed either in IU/l or as a

percentage, was higher in groups T and OT than in group C throughout the sampling period although the difference between groups T and C did not always reach statistical significance at weeks 10 and 11. The LDH_3 level in group OT tended to be higher than in group T but only at week 10 was the difference statistically significant.

From Table 9.6, total serum creatine kinase was, in general, higher in infected than control animals. The higher total enzyme level was due to an increased absolute value of the MM_2 isoenzyme and at weeks 10 and 13, to a higher absolute MM_2 and MB_1 level. These trends were not evident when the results for creatine kinase isoenzymes were expressed as a percentage of the total activity.

In order to determine whether the levels of the parameters which were fairly consistently higher in the infected groups [LDH_3 (IU/l and %), total creatine kinase and CK-MM_2(IU/l)] changed with time over the sampling period, paired t-tests were carried out to compare the results for individual animals at each week of sampling with the other three weeks (results not shown). LDH_3 (IU/l) was significantly higher at week 13 than week 10 for group T ($p < 0.05$) but there was no change with time in group OT. The percentage of LDH_3 did not vary with time in either group. Total serum creatine kinase in group T was higher at week

10 than at weeks 11 and 12 whereas in group OT the level was higher at weeks 10 and 13 than at week 11. Creatine kinase MM_2 (IU/l) was higher at weeks 10 and 12 than week 11 ($p<0.05$ and $p<0.01$ respectively) in group T and higher at week 10 than week 13 ($p<0.05$) in group OT.

The differences in serum LDH_3 and MM_2 levels between groups are illustrated in Figs. 9.1 and 9.2 .

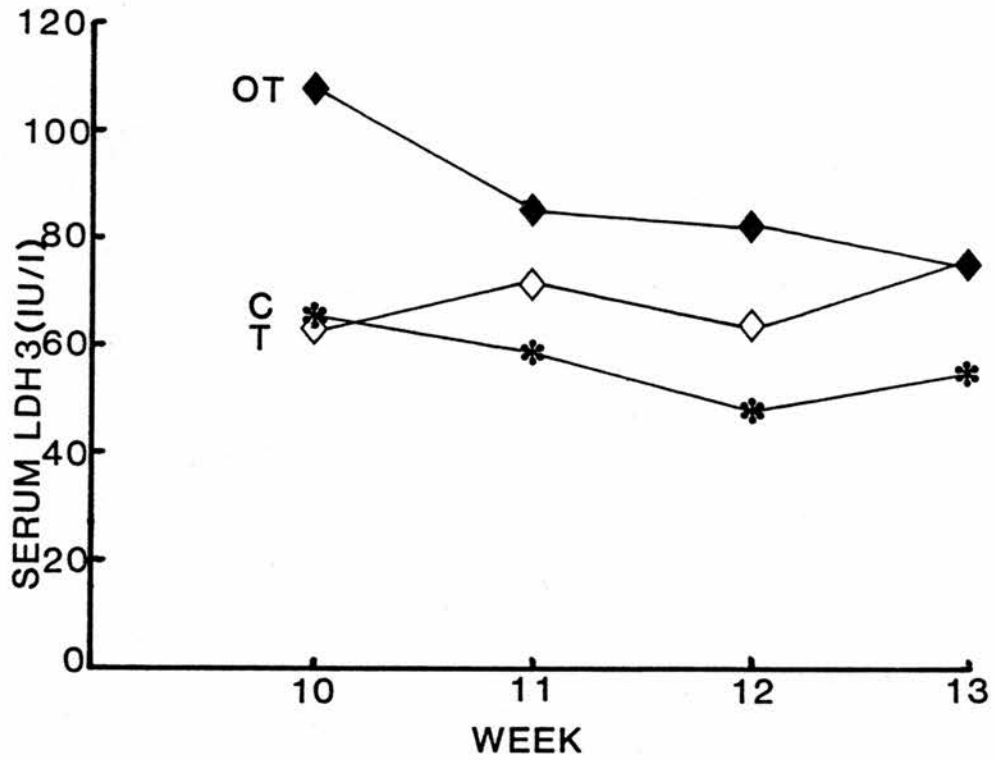
Mean values and standard deviations were calculated for total lactate dehydrogenase and its isoenzymes in the abomasal and small intestine mucosa of lambs in groups T and OT (Tables 9.7 and 9.8) and the results compared by Student's t-test.

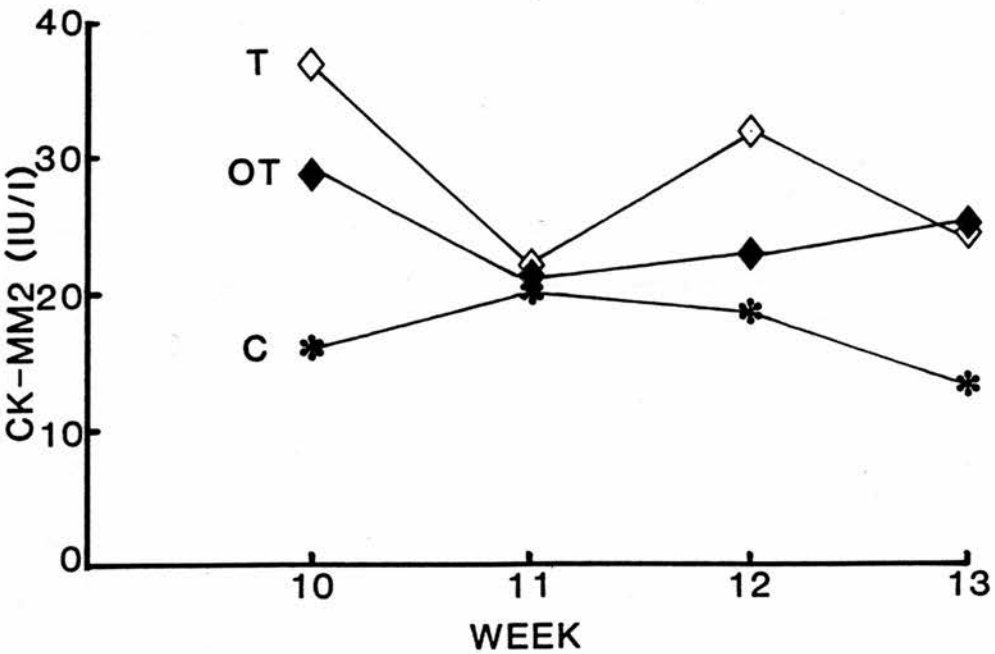
The results of Student's t-test (results not shown) indicated that there was no significant difference in the level of total lactate dehydrogenase or its isoenzymes in the abomasal mucosa between groups T and OT. Only minor differences were observed in isoenzyme levels in the small intestine mucosa, group OT having significantly higher absolute and percentage levels of LDH_1 and LDH_2 ($p < 0.01$ and $p<0.05$, respectively) than group T. The total enzyme level in the small intestine did not differ significantly between groups.

Ideally, the levels of total lactate dehydrogenase and its isoenzymes in the abomasal and small intestine mucosae of groups T and OT should have been compared

Figure 9.1. Serum LDH_3 in lambs with chronic infections with Trichostrongylus vitrinus (T), T. vitrinus and Ostertagia circumcincta (OT) and in worm-free control lambs (C) from 10-13 weeks after initial infection (Chapter 9, Part 1).

Figure 9.2. Serum creatine kinase MM_2 in lambs with chronic infections with T. vitrinus (T), T. vitrinus and O. circumcincta (OT) and in worm-free control lambs (C) from 10-13 weeks after initial infection (Chapter 9, Part 1).


120
100
80
60
40
20
0
SERUM LDH 3(IU/l)
OT
C
T
10
11
12
13
WEEK


40
30
20
10
0
CK-MM2 (IU/l)
T
OT
C
10
11
12
13
WEEK

with those of group C, but for reasons which have already been stated, tissues were not available from the control animals. The levels in the infected lambs were therefore compared with those obtained in normal lambs of the same age (see Chapter 6, Part 2). The results are shown in Table 9.9.

In the abomasal mucosa, total lactate dehydrogenase was significantly higher in normal lambs than in group T. In both infected groups, the absolute and percentage LDH_1 levels were lower and LDH_3, LDH_4 and LDH_5 higher than in normal animals. In the small intestine mucosa, total lactate dehydrogenase levels did not differ between normal and infected lambs, and few significant differences in the isoenzyme levels were observed, although the absolute and percentage level of LDH_1 was higher in normal lambs than lambs in group T and LDH_4 lower in normals than in group OT.

During the course of the infection, a few animals showed soft faeces for several days but none showed signs of scouring. This was also observed, albeit to a lesser extent, in the control group and was considered to be partly dietary in origin (Coop, 1982).

Group T and OT showed three peaks in their faecal egg count at 3 weeks, 6 weeks and 10 weeks after the initial infection. Approximate mean egg counts were as follows:

3 weeks - Group T 250 eggs per gram (epg)

Group OT 350 epg

6 weeks - Group T 600 epg

Group OT 900 epg

10 weeks - Group T 300 epg

Group OT 300 epg

Control lambs had no eggs in the faeces.

At slaughter, total worm burdens in the entire small intestine were estimated in an aliquot of diluted small intestinal contents. Mean worm burdens were 12,141 (range 196-25,420) in group T and 4745 (range 30-13,320) in group OT. Macroscopic examination of the upper small intestine revealed localised areas of flattened mucosa ("fingerprint" lesions) or more generalised flattening in both infected groups, while abomasa in group OT only, showed hyperaemia, thickening of the mucosa and the presence of numerous pale nodules. Abomasal worm counts were not available at the time of writing.

To determine whether there was a significant correlation between the parameters which were fairly consistently higher in infected than control animals (i.e. LDH_3 in IU/l, $\%LDH_3$, total creatine kinase and MM_2 in IU/l), and total worm burdens at slaughter, linear regression analysis was carried out. In group T, no significant correlations were found at weeks 10, 11, 12 and 13 after infection, but in group OT, total

small intestinal worm burden showed a significant positive correlation with total serum creatine kinase at 12 weeks after initial infection ($r = 0.828$; $df = 5$; $p < 0.05$).

DISCUSSION

Trichostrongylus vitrinus is a common parasite of the small intestine in sheep in Britain and is more prevalent than T. colubriformis (Reid and Armour, 1975), although the latter has been more extensively studied. The prepatent period is three weeks following the ingestion of infective third stage larvae, with a peak of egg production 5-6 weeks after infection. Chronic infection may produce thickening and inflammation of the mucosa in the first 5-7 metres of the small intestine with bright red, sharply demarcated areas of mucosa, devoid of villi ("fingerprint" lesions) which may become confluent. Less severely affected animals show subtotal villous atrophy and crypt elongation (Coop, Angus and Sykes, 1979). A total worm count of 4000 Trichostrongylus species in the small intestine is considered to be the minimum pathogenic level in lambs subject to factors such as the state of nutrition (Doxey, 1971). In the present study, the administration of 2000 T. vitrinus larvae daily for 13 weeks produced typical lesions in groups T and OT, with total small intestine worm burdens exceeding 4000 in 5 lambs in group T and 4 lambs in group OT.

Ostertagia circumcincta inhabits the abomasum of sheep. After ingestion of third stage larvae, the parasites enter the crypts of the abomasal glands where they develop into fourth stage larvae. Since the fourth stage larvae are the most pathogenic stage, faecal egg counts are of little use in diagnosis. Following larval emergence from the glands, the mature worms attach to the surface of the mucosa. The prepatent period is 2-3 weeks (Coop, Sykes and Angus, 1977). Invasion of the abomasal glands produces mucosal oedema and the formation of a small pale nodule at the site of each larva and larval emergence causes sloughing of the mucosa. The pathological changes in O. circumcincta infection will be described in detail in Part 2.

The administration of 1500 O. circumcincta larvae daily for 13 weeks resulted in the formation of numerous nodules but the abomasal worm burdens were not available at the time of writing. A minimum level of 3000 worms may be pathogenic in lambs (Doxey, 1971).

The fact that clinical signs were not observed in either infected group may be due to the good nutritional status of the lambs before and during the experiment, and the doses of larvae given were relatively small under such circumstances. Nevertheless, subclinical parasitism is known to have marked effects on blood chemistry, for example Coop, Sykes and Angus (1976)

found hypophosphataemia, hypoalbuminaemia and hyperglobulinaemia in subclinical trichostrongylosis and Coop, Sykes and Angus (1977) demonstrated hypoalbuminaemia in subclinical O. circumcincta infection.

A number of reports describe elevations in serum enzyme or isoenzyme levels in chronic gastrointestinal tract disease. Examples include raised serum alkaline phosphatase levels in intractable diarrhoea in cattle (Healy, 1971), the appearance of an additional alkaline phosphatase band in Crohn's disease and ulcerative colitis in man (Qirbi and Moss, 1975), increased "intestinal" alkaline phosphatase in intestinal parasitism in horses (Blackmore and Palmer, 1977) and elevated serum pepsinogen levels in ostertagiasis in calves (Mylrea and Hotson, 1969) and lambs (Armour, Jarrett and Jennings, 1966; Coop, Sykes and Angus, 1977).

In the present study, lambs infected with both T. vitrinus and O. circumcincta consistently showed higher absolute and percentage levels of LDH_3 in the serum, compared with the control animals. Lambs infected with T. vitrinus alone showed a similar trend. The isoenzyme distribution in small intestine mucosa (see Table 9.8) confirmed the results obtained in normal lambs (see Chapter 6, Part 2) indicating that LDH_3 forms the greatest proportion of the lactate dehydrogenase activity and with the exception of LDH_1,

which showed a highly significant difference between normal and group T lambs, the isoenzyme distribution in the small intestine mucosa was similar in lambs in the present experiment and the previously established normal values (see Table 9.9). Elevations in the serum LDH_3 level are therefore consistent with release from damaged small intestine mucosa. No consistent significant differences were observed in serum lactate dehydrogenase or its isoenzymes between the two infected groups and it was concluded that O. circumcincta infection contributed little to the altered serum LDH_3 level in group OT. In the abomasum, LDH_1 was the predominant isoenzyme in both infected groups and in normal lambs but a higher serum level was not observed in group OT compared with the control group. This tends to substantiate the view that detectable amounts of lactate dehydrogenase are not released into the serum in chronic ostertagiasis, particularly since the total enzyme activity in the abomasal mucosa is relatively high (almost twice the level in small intestine mucosa in this experiment) giving the abomasum a considerable potential for altering the serum lactate dehydrogenase activity.

The lactate dehydrogenase isoenzyme levels and percentage distribution in the abomasal mucosa differed markedly from the normal values obtained in Chapter 6, although LDH_1 still constituted the highest proportion

of the activity, followed by LDH_3. The higher activity of the more cathodal isoenzymes (LDH_3, LDH_4 and LDH_5) in the experimentally infected groups is unlikely to be due to changes in the cell types present in the abomasal mucosa in parasitic infestations since it was observed in group T lambs which were free from abomasal parasites. Many factors could account for the differences between the normal and experimentally infected lambs other than those due to parasitic infestations, particularly since the "normal" lambs were commercial sheep and therefore not worm-free. Such factors include breed and conditions of management (the normal lambs were at grass, whereas groups T and OT had been housed since birth). Clearly it would have been preferable to have compared the tissue isoenzyme levels in infected lambs with those of the control group.

Total serum creatine kinase tended to be higher in both infected groups than in the controls, largely as a result of an increase in the absolute MM_2 level but the level of the BB isoenzyme, which is present in by far the highest proportion of the five isoenzymes in the mucosa of the ovine gastrointestinal tract was not significantly different between infected and control lambs. MM_2, which is thought to be the mitochondrial isoenzyme, is detectable in small intestine mucosa, although it only constitutes approximately 0.5% of the

total activity, but is apparently not detectable in abomasal mucosa (see Chapter 6, Part 2). This would suggest that in both infected groups, the increased serum level is due to release from the small intestine mucosa. The two other organs in which MM_2 was detected i.e. liver and lung, were macroscopically normal and there was no evidence that the increased level was due to release from these sites. Thus, like LDH_3, it appears that MM_2 is released from the small intestine during chronic trichostrongylosis but not chronic ostertagiasis. The fact that serum creatine kinase BB was not raised in infected animals is difficult to explain in the presence of a raised serum LDH_3 level.

The failure of chronic ostertagiasis to cause elevations in serum isoenzymes due to release from the abomasal mucosa may be associated with the "self cure" mechanism. This phenomenon has been described by Coop, Sykes and Angus (1977), who noted that between 8 and 14 weeks after initial infection of lambs receiving daily doses of O. circumcincta, the worm population in the abomasum changed from a mixture of adults and fourth stage larvae to a predominance of the latter, with a reduction in their numbers, and loss of adult worms. Similarly, Gibson and Everett (1978) found that after 12 weeks of continuous dosing with O. circumcincta larvae, host resistance limited worm burdens and egg output. In my experiment lambs

were sampled from 10-13 weeks after initial infection and the resistance mechanism was likely to be active during this period, presumably resulting in partial recovery of the abomasal mucosa from parasitic damage and preventing further damage by the daily doses of larvae with a consequent lack of release of enzymes from the abomasal mucosa into the circulation. A second possibility for the failure to detect increased isoenzyme levels in chronic ostertagiasis is that they may be released in damage to the abomasal mucosa, but the dilution effect of the serum is such that increased serum levels are not detectable. Thirdly, sloughing of the abomasal mucosa may result in loss of cellular enzymes into the digestive tract rather than leakage into the circulation.

The potential value of serum LDH_3 and MM_2 measurements in the diagnosis of naturally occurring trichostrongylosis is questionable. The marked effect of age on the absolute and percentage levels of LDH_3 would have to be taken into account (see Chapter 6, Part 3) together with possible breed differences in the normal levels. It should be emphasised that the control animals in this experiment had been kept under worm-free conditions since birth and any differences in the parameters measured compared with those in the infected animals would undoubtedly be

more marked than had conventionally reared control sheep been used. Thus in field cases of trichostrongylosis, comparison of serum LDH_3 and MM_2 levels with those obtained in normal conventional lambs of the same age and breed, kept under similar conditions, may not provide evidence of raised levels in the affected lambs. One area where the measurement of these two isoenzymes could be of value, is in providing evidence for the existence of damage to the small intestine mucosa in the live animal with experimentally-induced trichostrongylosis, provided that comparison could be made with worm-free controls. This experiment however, has provided no clear evidence that the absolute or percentage levels of LDH_3, total serum creatine kinase or the absolute level of MM_2 show a consistent, significant correlation with the size of the worm burdens in chronic trichostrongylosis.

PART 2 - ACUTE AND CHRONIC STAGES OF PARASITIC GASTRITIS

Two experiments were designed to investigate serum isoenzyme levels over a period of 2-3 months after infection with abomasal parasites. In the first experiment, *Haemonchus contortus* larvae were used as a means of producing damage to the abomasal mucosa while the second involved larvae of *O. circumcincta*.

A. PARASITIC GASTRITIS DUE TO HAEMONCHUS CONTORTUS INFECTION

EXPERIMENTAL DESIGN

Nine, clinically normal, 6 month old Suffolk x Greyface lambs which had been reared under worm-free conditions were each given a single oral dose of 10,000 H. contortus larvae. Lambs were separated from a larger group on the day of infection and housed in one pen throughout the experiment. They were fed on ruminant A.

PARAMETERS INVESTIGATED

Serum was obtained immediately before infection and at weekly intervals thereafter for 12 weeks for estimation of total lactate dehydrogenase, total creatine kinase and their isoenzymes. The lambs therefore acted as their own controls. Samples of abomasal mucosa could not be obtained and abomasal worm counts and post-mortem examination could not be undertaken as the lambs were required for a subsequent experiment by the Moredun Institute.

MATERIALS AND METHODS

H. contortus larvae were from a strain which has been maintained in the Moredun Institute for several years by serial passage through sheep. Larvae were administered orally as an aqueous suspension.

Serum samples were collected, divided into two 100µl and one 1-2ml aliquot and stored as previously

described. Total enzyme and isoenzyme estimations were undertaken as before.

RESULTS

The mean serum enzyme and isoenzyme levels and their standard deviations were determined and the results presented in Tables 9.10 - 9.13. Paired t-tests were carried out to ascertain whether the levels changed over the 12-week period with respect to the pre-infection level. The t values and levels of significance are shown in Tables 9.14 and 9.15.

Differences in the level of lactate dehydrogenase isoenzymes with respect to the pre-infection level were mainly confined to weeks 5 to 9 after infection but these changes had little effect on the total enzyme level (Table 9.14). The absolute and percentage levels of LDH_1 tended to be higher, and LDH_2, $_4$ and $_5$ lower during this period than at week 0 although the differences were not significant for all isoenzymes at each week between 5 and 9. LDH_3 did not change throughout the experiment.

Total creatine kinase was significantly lower towards the end of the experiment than before infection (Table 9.15). In contrast to the results for lactate dehydrogenase isoenzymes, significant differences occurred in creatine kinase isoenzyme levels with respect to the pre-infection level throughout the twelve weeks. Statistical significance was more

often reached, and the level of significance tended to be higher when isoenzyme values were expressed as a percentage. Thus, the $\%MM_2$ and $\%MB_1$ isoenzymes increased and the $\%MM_1$ isoenzyme decreased from week 2. Only minor changes were observed in the BB isoenzyme level, with no evidence of any trend.

During the course of the infection, no clinical signs of H. contortus infection were seen.

B. PARASITIC GASTRITIS DUE TO OSTERTAGIA CIRCUMCINCTA INFECTION

EXPERIMENTAL DESIGN

Eight, clinically normal, 8 week old Suffolk x Greyface lambs which had been reared under worm-free conditions each received 2000 O. circumcincta larvae daily, 5 days per week for 52 days. Lambs were separated from a larger group on the day of infection and were kept in one pen throughout the experiment. They were fed on ruminant A.

PARAMETERS INVESTIGATED

Serum was collected immediately before administration of the first dose of larvae and twice weekly thereafter for 7½ weeks. Serum was analysed for total lactate dehydrogenase, total creatine kinase and their isoenzymes. As in the previous experiment, post-mortem examination and abomasal worm counts could not be carried out and samples of abomasal mucosa could not be collected as the lambs were required for another

experiment.

MATERIALS AND METHODS

O. circumcincta larvae were from a strain which has been maintained in the Moredun Research Institute for several years by serial passage through sheep.

Serum samples were collected, divided into aliquots, stored and analysed for total enzyme and isoenzyme levels as previously described.

RESULTS

The mean values and standard deviations of total serum lactate dehydrogenase, total creatine kinase and their isoenzymes were calculated from the data from each of the eight lambs, at each sampling. As in the previous experiment, paired t-tests were carried out to compare the levels at day 0 with those obtained at each of the subsequent samplings. The mean values are given in Tables 9.16 - 9.19 and the t values and levels of significance in Tables 9.20 and 9.21.

Neither the total lactate dehydrogenase nor the creatine kinase level was significantly different from the pre-infection level throughout the experiment. When expressed in IU/l, LDH_1 was significantly lower than the pre-infection level from day 28 and LDH_2 tended to be lower from day 38 but the other isoenzymes showed no change. When expressed as a percentage however, only minor differences were observed in LDH_1,

LDH_2 and LDH_5, but LDH_3 was higher from day 35 and LDH_4 tended to be lower from day 10.

Creatine kinase MM_2 in IU/l, was in general higher than the pre-infection level from day 21 and the BB isoenzyme from day 31 but no changes were observed in the absolute MM_1 or MB_1 levels. Percentage values however, showed a clearer trend with MM_2 and BB increased and MM_1 decreased from day 7 with little change in MB_1 percentage.

Clinical signs of ostertagiasis were not observed although soft faeces were passed from days 17 to 35.

DISCUSSION (EXPERIMENTS A AND B)

Haemonchus contortus is an extremely pathogenic abomasal parasite of sheep. Following ingestion, the infective third stage larvae migrate to the abomasum where they mature in 19 days. The pathogenic effect is due to the blood-sucking activities of the fourth stage larvae and the adult worms. In acute cases in young sheep, death may occur rapidly with anaemia being the only finding whereas in chronic cases, the anaemia is accompanied by ventral oedema, wasting and weakness. In severe infections, the abomasal mucosa is oedematous and hyperaemic with the presence of many pin-point haemorrhages. In lambs, a total abomasal worm count of 500 H. contortus is considered to be the minimum level likely to be pathogenic (Doxey, 1971). The single oral dose of 10,000 larvae

in experiment A was not a heavy challenge and no clinical signs of haemonchosis were observed.

In experiment B, daily doses of 2000 O. circumcincta larvae, a relatively low level of infection, produced softening of the faeces during the time when the infection was becoming patent, but no other clinical signs of ostertagisis. Unfortunately, since the lambs in both investigations were required for a further experiment, they were not slaughtered and it was not possible to obtain data on the worm burdens present or to determine the extent of damage to the abomasal mucosa.

In the previous experiment (Chapter 9, Part 1), increases in serum LDH_3 and MM_2 were observed in chronic T. vitrinus infection but apparently not as a result of the ostertagia component of a chronic mixed T. vitrinus and O. circumcincta infection. Experiments A and B were designed to investigate whether serum isoenzyme changes occurred at any stage in the course of infection with moderate levels of abomasal parasites.

Serum enzyme levels in the acute stages of various gastrointestinal diseases have been reported, the majority referring to intestinal rather than gastric involvement. Examples include studies on total serum lactate dehydrogenase in intestinal strangulation and infarction in man (Ticktin and Trujillo, 1970), total

serum lactate dehydrogenase and creatine kinase and their isoenzymes in acute colonic infarction in dogs (Graeber et al., 1981) and serum creatine kinase isoenzymes in gastrointestinal tract surgery in man (Tsung, 1981). Graeber et al., (1981) found a differential increase in LDH_3, the predominant isoenzyme in canine human bowel, but the increase in total creatine kinase was mainly due to an increase in MM, which does not predominate in bowel. Similarly, Tsung (1981) found MB to be raised more often than BB, although the latter is the predominant isoenzyme in the intestine. Increases in serum or plasma pepsinogen levels in ostertagiasis of sheep and cattle have been reported and will be discussed in Part 3.

In order to interpret the changes in total serum enzyme and isoenzyme levels in experimental H. contortus infection in experiment A, it is necessary to consider the time-scale of these changes in relation to the events occurring in the abomasum, i.e.

(a) the "self-cure" mechanism is maximal at 10-14 days after a single oral dose of larvae in previously worm-free lambs.

(b) by day 6, the majority of larvae have emerged from their site of development in the mucosa and are found in the lumen.

(c) damage to the mucosa starts at day 4 and continues at least until day 33 (Malczewski, 1971).

Thus, it appears that the changes in serum lactate dehydrogenase isoenzymes which occurred between weeks 5 and 9, did not coincide with the period of maximal numbers of larval or adult worms, nor with emergence of the majority of larvae from the mucosa. By contrast, changes in the creatine kinase isoenzyme distribution, which were evident after week 1, occurred just after the onset of tissue damage and larval emergence but it cannot be said with certainty that these events caused the isoenzyme changes, particularly as they were still evident long after the expected time for the "self-cure" mechanism to produce its maximum effect with a subsequent recovery of the damaged mucosa.

Although the time of onset and duration of changes in serum isoenzyme levels are difficult to reconcile with the changes occurring in the abomasum, several points can be made regarding the nature of the isoenzyme changes.

(1) The increased percentage of serum LDH_1 in the presence of an increased absolute LDH_1 level is compatible with its release from absomasal mucosa where it is the predominant lactate dehydrogenase isoenzyme, at least in normal lambs (see Chapter 6, Part 2) and lambs with ostertagiasis (see Chapter 9, Part 1), but unfortunately it was not possible

to determine the isoenzyme distribution in the mucosa of the lambs in this experiment. The absolute LDH_1 activity in abomasum is also high and is second only to heart muscle (see Chapter 6, Part 2).

(2) The absolute MM_1 level fluctuated markedly during the course of the experiment with resulting fluctuations in the total creatine kinase level (see Table 9.12).

In the normal lambs in Chapter 8, Part 1 (see Table 8.4) the mean values for MM_1 ranged from 11-27 IU/l compared with a range of 14-98 IU/l in the present experiment, with values of 56 and 98 IU/l at weeks 0 and 1 respectively. Thus, the observation that the absolute and percentage MM_1 level was generally <u>lower</u> than the pre-infection level from week 2 onwards was considered to be due to the levels at weeks 0 and 1 being abnormally high, probably as a result of minor trauma to skeletal muscle in which this isoenzyme predominates. Such trauma may have occurred during movement into new accommodation at the start of the experiment, while the fluctuations in serum MM_1 levels throughout the experiment was probably associated with minor trauma during handling of the lambs which tended to struggle when restrained.

(3) The increase in the percentage of MB_1 was not solely due to a reciprocal decrease in the percentage of MM_1 since significant increases in the absolute level of MB_1 were also consistently observed but the reason for this increase is obscure since the highest absolute levels of this isoenzyme are found in small and large intestine.

(4) In contrast to (3), the increase in the percentage of MM_2 was only occasionally reflected in an increased absolute level, suggesting that the increased percentage was probably reciprocal to a decreased MM_1 percentage.

(5) Creatine kinase BB level showed no trend with time after infection although it is the predominant isoenzyme in abomasal mucosa.

In ovine O. circumcincta infection due to a single dose of 100,000 larvae, the time-scale of the parasitological and pathological changes have been described by Armour, Jarrett and Jennings (1966). Clearly, the pathological changes may differ in infections due to single and multiple doses of larvae, but in the apparent absence of information on the effect of multiple doses, the report by Armour, Jarrett and Jennings will serve as a basis for correlating the enzyme changes with those occurring in the abomasum. They found that most larvae had entered the gastric glands by day 4 resulting in hyperplasia of the

epithelium of occupied glands and elongation of unoccupied glands. At 8 days, some larvae had emerged from the glands but those remaining caused marked distention of the glands, which were lined by undifferentiated epithelium. Local areas of submucosal oedema were present. By day 12, severe hyperplasia of the epithelium had developed and there was evidence of a foreign body reaction where larvae had emerged. A range of lesions was present at 16 days due to development of larvae which had previously been inhibited. Cytolysis was evident in the lesions from which larvae had emerged, causing sloughing and pitting of the mucosa. This coincided with a decrease in the adult worm population due to the "self-cure" mechanism. At 21-28 days, the upper part of the larvae-containing glands had sloughed. By 35 and 60 days the lesions were regressing but pitting of the mucosa and lesions due to larval emergence were still evident. Some inhibited larvae were still present in the mucosa at day 60, but abomasal function had returned to normal.

The changes in serum isoenzyme levels in experiment B will now be considered in relation to the expected times of the events in the abomasum.

(1) The absolute LDH_1 level was lower than the pre-infection level from day 28 which coincided with

mucosal sloughing. The reason for a decrease in LDH_1 is uncertain but the fact that it did not increase may be associated with loss of the abomasal mucosa in which it is the predominant isoenzyme, thereby preventing leakage of the isoenzyme from damaged cells into the circulation. Alternatively, a decreased serum LDH_1 level could result from a decrease in LDH_1 activity in the mucosa in ostertagiasis. The latter will be discussed in Part 3.

(2) The percentage of LDH_4 was lower from day 10, approximating to the time of maximal damage to the glands by the presence of larvae within them whereas the percentage of LDH_3 was higher from day 35 when the lesions were starting to regress.

(3) As in the previous experiment, the mean values for total serum creatine kinase and the absolute and percentage MM_1 levels were abnormally high at days 0 and 3 but owing to the very marked individual variation on these days (Tables 9.18 and 9.19), the absolute levels of these two parameters were not significantly different from the pre-infection level throughout the experiment. The percentage of MM_1 however, was significantly lower than the pre-infection level from day 7 onwards. Again, the initially

high MM_1 level was considered to be due to the trauma of handling and moving accommodation on day 0 and in this experiment, this proposal was confirmed by an abnormally high mean total serum lactate dehydrogenase and LDH_5 level at days 0 and 3 (Tables 9.16 and 9.17), LDH_5 predominating in most skeletal muscles.

(4) The percentage of MM_2 was higher from day 7 but this was only reflected in its absolute level from day 21, the time of onset of mucosal sloughing. The increased absolute level continued although abomasal lesions would be regressing. The reason for the increase is difficult to explain since MM_2 is only detected in liver, lung and small intestine homogenates.

(5) The percentage of BB was also higher from day 7 and was reflected in a higher absolute level from day 35 when the lesions were starting to regress. The increased serum BB is consistent with release from damaged abomasal mucosa where it is the predominant isoenzyme. However, it is odd that the predominant lactate dehydrogenase isoenzyme in mucosa did not increase in the serum coincidentally with BB.

Comparison between the results obtained in experiments A and B reveals few similarities, but the differences should not be solely attributed to the different parasites used since in one experiment, a

single dose of larvae was given while in the second, the lambs were dosed repeatedly. However, the increase in serum LDH_1 in experiment A and decrease in experiment B may have occurred because sloughing of the LDH_1 - rich mucosa into the abomasal lumen is more of a feature of O. circumcincta than H. contortus infection or because O. circumcincta infection causes a decrease in the LDH_1 level in the mucosa. An increase in the percentage of MM_2 occurred in both experiments but only in experiment B was it associated with an increased absolute level. In both experiments, MM_1 was abnormally high at the start, probably due to minor trauma during handling. In experiment A, this caused a reciprocal increase in the percentage of MM_2 when MM_1 returned to normal. In experiment A, MB_1 increased and BB was unchanged whereas in experiment B, BB increased and MB_1 was unchanged. The interference of increased MM_1 levels due to minor trauma appears to be a hazard of following the course of a disease under experimental conditions as it was observed in both experiments and in one of the experiments in the previous chapter.

The results suggest that none of the serum isoenzymes can be recommended as an aid in the detection of "abomasal damage" in general, since the results varied markedly between the two experiments. Although MB_1 was fairly consistently increased in H. contortus infection and BB in O. circumcincta infection, the

value of such measurements in mixed, field infections is questionable.

PART 3 - ACUTE PARASITIC GASTRITIS

The three preceeding experiments indicated that few consistent changes were detectable in total or isoenzyme levels of lactate dehydrogenase and creatine kinase during parasitic damage to the abomasal mucosa. The dilution effect of the serum on cellular enzymes released during tissue damage was considered to be a contributory factor.

The object of this experiment was to investigate whether enzymes are in fact released into the serum from the abomasal mucosa during acute parasitic gastritis but do not show detectable increases in their serum activity. This was accomplished by the measurement of enzyme levels in lymph obtained directly from the local lymphatic drainage from the abomasum.

EXPERIMENTAL DESIGN

Four clinically normal, 10 month old, Suffolk x Greyface sheep which had been reared and housed in worm-free conditions underwent surgery for cannulation of the common gastric lymph duct. Three days later each were given a single oral dose of 50,000 *O. circumcincta* larvae. After surgery, the sheep

were housed in individual pens and fed ruminant A, ad lib. Twenty-one days after administration of larvae, the sheep were slaughtered.

PARAMETERS INVESTIGATED

Lymph samples were obtained immediately before infection and every second day thereafter until day 20 post-infection. Serum samples were collected from each sheep on five occasions after infection i.e. on days 2 - 5, 9 - 11, 12 - 14, 16 - 18 and 20 - 21. Unfortunately it was not possible to obtain pre-infection serum samples, nor to obtain serum on the same days after infection for each sheep since the experiment was staggered so that each sheep was infected on a different day and it was impossible to gain access to them all at the same time intervals after infection. For simplicity, serum sampling times will hereafter be referred to as days 3, 10, 13, 17 and 20. Total lactate dehydrogenase, total creatine kinase and their isoenzymes were estimated in both lymph and serum and total protein and pepsinogen levels were estimated in lymph. At slaughter, adult and larval worm burdens in the abomasa were counted and abomasal mucosa was collected for estimation of total lactate dehydrogenase, total creatine kinase and their isoenzymes. Lymph pepsinogen, lymph flow rates and worm burdens were determined and surgery carried out by staff of the

Moredun Research Institute.

MATERIALS AND METHODS

The procedure for cannulation of the common gastric lymph duct has been described in detail elsewhere (Smith et al., 1981). Briefly, animals were anaesthetised with a halothane-nitrous oxide - oxygen mixture, the peritoneal cavity was opened on the right side behind the last rib and the abomasum was exposed. A few hundred µl of 1% Evans blue was injected subserosally into the abomasum and was taken up by the lymphatic system so that the gastric lymph duct appeared as a blue vessel running over the pancreas. The duct was ligated and cannulated with a polyvinyl chloride cannula which was led out through an incision high on the right flank and connected to a plastic reservoir bag attached to the flank. A second catheter was inserted in the jugular vein.

The preparation of O. circumcincta larvae for oral administration was as previously described. Lymph samples were collected directly from the cannula in two, 0.5ml microcentrifuge tubes while venous blood samples were collected from the catheter in the jugular vein for harvesting of serum, 0.5ml of which was then placed in a microcentrifuge tube. Daily lymph flow rates were calculated in ml/hr. from the volume of

lymph which accumulated in the reservoir bag over a known time period, which was usually 24 hours.

Total lactate dehydrogenase, total creatine kinase and their isoenzymes were determined in serum, lymph and abomasal mucosa as previously described. In order to take into account variations in lymph flow, enzyme and isoenzyme levels in lymph (except pepsinogen) were converted to IU/g protein in the lymph x 10. Lymph protein was determined by the biuret method as described in Chapter 6, Part 2. Lymph pepsinogen levels were determined by a modification of the method of Mylrea and Hotson (1969) and expressed in IU/l/min.

Adult worm burdens were estimated after slaughter in an aliquot of diluted abomasal contents, and numbers of larvae embedded in the mucosa were determined after digesting the abomasal wall with pepsin-HCl and counting the worms in an aliquot of the solution thus obtained. This procedure has been described by Coop, Sykes and Angus (1977).

RESULTS

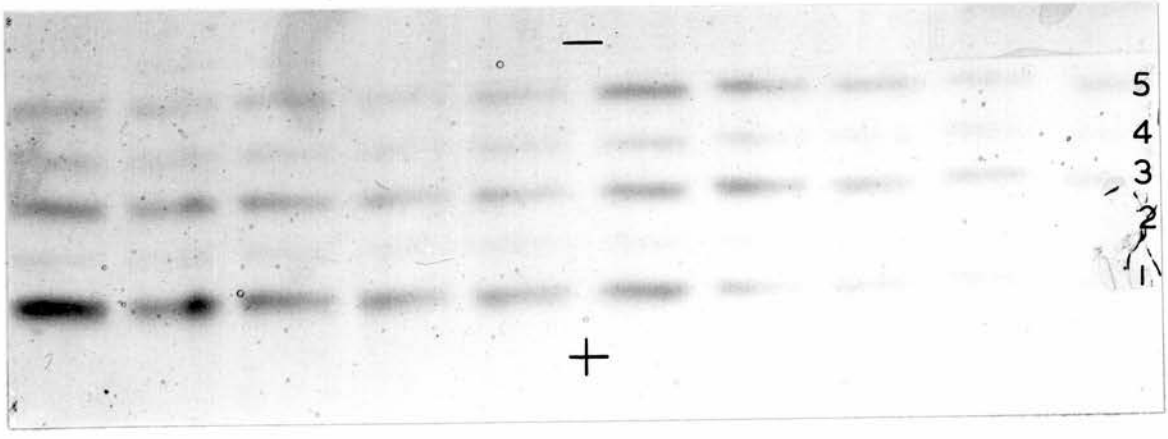
The mean values and standard deviations for total lactate dehydrogenase, total creatine kinase and their isoenzymes in serum and lymph and pepsinogen levels in lymph were calculated. The results are shown in Tables 9.22 - 9.25, 9.28 - 9.31 and 9.34. Since pre-infection serum samples were not obtained, paired t-tests

were used to compare the total enzyme and isoenzyme levels in serum at each sampling with the values at all other samplings. In lymph however, paired t-tests were carried out to compare the individual values of the above parameters at each sampling with the pre-infection levels. The results are presented in Tables 9.26 and 9.27, 9.32, 9.33 and 9.34. Typical lactate dehydrogenase zymograms from lymph samples are shown in Fig. 9.3.

In serum, no significant change in the levels of total lactate dehydrogenase nor creatine kinase occurred throughout the post-infection period. Few significant changes in their isoenzyme levels occurred and no distinct pattern of changes emerged although LDH_5 (IU/l and %) and the percentage of MM_2 tended to be higher and the percentage of LDH_1 and BB lower towards the end of the experiment. By day 20, the percentage distribution of creatine kinase isoenzymes had changed so that MM_2 showed a slightly higher activity than BB, the latter being the predominant isoenzyme up to day 17.

In lymph, little change in total lactate dehydrogenase and no change in total creatine kinase occurred throughout the experiment with respect to the pre-infection levels. The absolute isoenzyme levels of both enzymes also showed few significant changes but when expressed as a percentage, LDH_2 was fairly

Figure 9.3. Lactate dehydrogenase zymograms in lymph samples after separation by polyacrylamide gel electrophoresis and tetrazolium staining. (Chapter 9, Part 3) 1-5 = LDH_1 - LDH_5.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1

consistently higher than the pre-infection level from day 4 after infection as was creatine kinase BB from day 2, while MM_1 tended to be lower than the pre-infection level, mainly during the first 10 days after infection.

The mean lymph pepsinogen level reached a peak at 18 days after infection and was significantly higher than the pre-infection level at day 2 and from days 12 to 20.

The distribution of lactate dehydrogenase and creatine kinase isoenzymes in serum and lymph were compared at each of the time intervals during which serum samples were collected from the four sheep i.e. 2 - 5, 9 - 11, 12 - 14, 16 - 18 and 20 - 21 days post-infection, by paired t-tests. Each pair of samples was collected on the same day or within 24 hours of each other. The t values and levels of significance are shown in Tables 9.35 and 9.36.

Although the differences were not significant at each time interval, % LDH_1 tended to be higher and %LDH_4 lower in serum than in lymph and on two occasions, %LDH_5 was also significantly lower in serum. The percentage of creatine kinase MB_1 was significantly higher in lymph than in serum but there were no consistent differences in the distribution of the other three isoenzymes.

The means and standard deviations were calculated for total lactate dehydrogenase and creatine kinase

and the absolute and percentage levels of their isoenzymes in abomasal mucosa and the results compared with those obtained in normal lambs (see Chapter 6, Part 2) by Student's t-test. The results are shown in Tables 9.37 and 9.38 and the t values and levels of significance in Tables 9.39 and 9.40.

Total lactate dehydrogenase was significantly lower in the abomasal mucosa of infected than in normal lambs (Table 9.39) due to a significantly lower LDH_1 level. The percentage of LDH_1 was lower and the percentage of the other four isoenzymes higher in infected than normal lambs. Neither total creatine kinase nor its isoenzymes differed between normal and experimentally infected abomasa (Table 9.40). Only two creatine kinase isoenzymes, MM_1 and BB were present, as in normal abomasum.

Throughout the experiment the sheep showed no clinical signs of O. circumcincta infection. At slaughter, larval and adult abomasal worm burdens were estimated in the four sheep. The means and standard deviations were as follows:

adult worms 9675 $\pm$ 6207 (range 5709 - 18833)

larvae 1670 $\pm$ 1010 (range 701 - 3014)

total 11345 $\pm$ 7051 (range 6840 - 21847)

Investigations into the possible correlation between total enzyme or isoenzyme levels and worm burdens were not undertaken in view of the small number

of animals.

At post-mortem examination, the abomasa from all four sheep showed thickening of the mucosa, diffuse hyperaemia and the presence of numerous small white nodules.

DISCUSSION

Inoculation with a large number of O. circumcincta larvae is required to induce clinical signs of ostertagiasis, and Armour, Jarrett and Jennings (1966) failed to produce clinical signs after dosing sheep with twice the number of larvae used in this experiment. However in this study, the total abomasal worm count was well above the arbitrary value of 3000 worms which is considered to be the minimum number likely to be pathogenic (Doxey, 1971) and although the infection was subclinical, evidence of parasitic damage to the abomasal mucosa was found, as expected, at post-mortem examination.

A single dose of 50,000 O. circumcincta larvae produced no effect on total serum lactate dehydrogenase or creatine kinase, in agreement with the previous experiment involving repeated doses of 2000 larvae. Few significant changes in the absolute serum isoenzyme levels occurred although there were some changes in the percentage distribution, the predominant isoenzymes in intestinal mucosa (LDH_1 and BB) tending to be

lower towards the end of the experiment. In the previous experiment, a decrease in the absolute level of serum LDH_1 was observed. Although there was little change in the absolute level of serum LDH_1 in this experiment, the level of this isoenzyme in the abomasal mucosa was significantly lower than in mucosal samples from normal sheep (see Chapter 6, Part 2). This observation tends to substantiate the proposal that in the previous experiment, in which tissue samples were not analysed, the lower absolute serum LDH_1 level may have been due to a change in the isoenzyme distribution in the mucosa. However, in the present study, this does not appear to be the case since the absolute serum LDH_1 level showed little change throughout the experiment. Similarly, although the percentage of serum creatine kinase BB decreased towards the end of this experiment, the absolute level was unchanged. These findings stress the importance of measuring absolute serum isoenzyme levels in addition to percentage levels although the majority of reports discuss only changes in the latter. As in the preceeding experiment, an increase in the percentage of MM_2 in serum was observed. In the previous study the absolute MM_2 level was also higher than the pre-infection level from day 21, but in the present experiment serum samples were only collected until day 20. The

only serum isoenzyme to show a significant increase in its absolute level was LDH_5. This was unlikely to have originated from skeletal muscle since the absolute MM_1 level, a sensitive indicator of skeletal muscle damage, did not increase concomitantly. Small intestine mucosa has the second highest absolute and percentage level of LDH_5 but there was no evidence of small intestinal damage at post-mortem examination and the source of the LDH_5 is therefore unknown.

In lymph samples obtained from the local lymphatic drainage from the abomasum the results were similar to those of serum in that total lactate dehydrogenase and creatine kinase and the absolute levels of their isoenzymes showed few significant changes throughout the experiment. This indicated that analysis of lymph total enzymes and isoenzymes was not a more sensitive indicator of damage to the abomasal mucosa than serum total enzyme and isoenzyme levels, and the isoenzyme distribution in lymph from the abomasal lymphatic drainage did not resemble the distribution in abomasal mucosa more closely than the serum isoenzyme pattern. The only changes in lymph isoenzyme levels were in the percentages of LDH_2 and BB which were generally significantly higher and MM_1 which was generally lower in comparison with the pre-infection levels but little importance can be attached to these findings since they were not

reflected in significant differences in the absolute isoenzyme levels in IU/g lymph protein.

Serum or plasma pepsinogen levels are frequently estimated in ostertagiasis in ruminants. Mylrea and Hotson (1969) found that in previously worm-free calves under experimental conditions, serum pepsinogen was related to the intake of O. ostertagi larvae, but in field cases, the activity was unrelated to faecal egg counts or worm burdens, and varied with age. In sheep, Armour, Jarrett and Jennings (1966) reported an increase in plasma pepsinogen to twice the pre-infection level within 7 days of infection of previously worm-free animals with O. circumcincta. The increase continued until day 16, followed by an exponential decline. Coop, Sykes and Angus (1977) found elevated serum pepsinogen concentrations during larval emergence from the abomasal glands in experimentally induced subclinical ovine ostertagiasis associated with impaired growth. In the naturally occurring disease, elevated levels are reported in housed (Reid and Armour, 1973) and grazing sheep (Reid and Armour, 1975). In the latter report, the authors stated that serum pepsinogen estimations provide a useful guide to the degree of abomasal damage present in grazing sheep. Similarly, Thomas and Waller (1975) found pepsinogen levels to be correlated with Ostertagia burdens in grazing lambs

and stated that the pepsinogen level is directly related to abomasal damage and is a much earlier indication of worm build up than faecal egg count.

In the present experiment, pepsinogen levels were measured in lymph draining from the abomasal mucosa. The mean levels in the four sheep showed a gradual increase to day 18, the levels being significantly higher than the pre-infection level from day 12 to day 20 which was in close agreement with the peak at 16 days reported in serum by Armour, Jarrett and Jennings (1966). The subsequent decline in the pepsinogen level, which commenced at day 20 in my experiment, was considered by Armour, Jarrett and Jennings to be associated with a decline in the leakage of macromolecules although there was no histological evidence of regression of the lesions in the abomasal mucosa, and with the exponential loss of the worm burden. Thus, the present findings indicate that lymph pepsinogen, like serum pepsinogen appears to be a good indicator of the time of larval emergence and loss of the Ostertagia burden due to host resistance.

No changes which were likely to be of diagnostic value occurred in the levels of total serum lactate dehydrogenase, total creatine kinase or their iso-enzymes. Since no such changes occurred in abomasal lymph it was concluded that during the acute phase of subclinical ovine ostertagiasis, leakage of these

enzymes from abomasal mucosa into the circulation via the lymphatic drainage does not occur or is at too low a level to be detectable. Leakage of cellular enzymes into the lumen of the abomasum may occur rather than leakage into the circulation, which would provide an explanation for the failure to detect any consistent changes in lactate dehydrogenase, creatine kinase or their isoenzymes in serum or lymph in this experiment.

CONCLUSIONS FROM CHAPTER 9

Total serum lactate dehydrogenase, creatine kinase and their isoenzymes were studied in subclinical gastrointestinal parasitism in sheep.

In chronic infection with T. vitrinus with or without concurrent O. circumcincta infection, serum LDH_3 and MM_2 were elevated and were considered to originate from the small intestine mucosa but were independent of the size of the worm burden. Serum enzyme changes attributable to the Ostertagia component of mixed infections were not detectable. The value of serum LDH_3 and MM_2 measurements in field cases of trichostrongylosis is questionable but they may be useful markers of small intestine damage in the live animal under experimental conditions.

In H. contortus infection followed through its acute and chronic stages, the absolute serum LDH_1

activity tended to be raised, which was consistent with its release from damaged abomasal mucosa, where it is the predominant lactate dehydrogenase isoenzyme but the predominant creatine kinase isoenzyme (BB) showed no trend. Serum MB_1 was also consistently raised but its source was uncertain. In the acute and chronic stages of O. circumcincta infection, the absolute serum LDH_1 level decreased. This may be associated with a decrease in the absolute LDH_1 level in the abomasal mucosa in ostertagiasis which was demonstrated in the subsequent experiment.

It was concluded that none of the isoenzymes were suitable for detecting abomasal damage in mixed field infections since the results varied markedly with the cause of the damage.

In acute O. circumcincta infection, serum isoenzyme measurements were of no value in detecting abomasal damage, and isoenzyme levels in lymph collected directly from the local lymphatic drainage from the abomasum were no more sensitive than serum levels, indicating that leakage from damaged mucosa into the circulation does not occur or is at too low a level to be detectable. The fate of the cellular enzymes in the damaged mucosa may be release into the lumen of the abomasum rather than the circulation, particularly since mucosal sloughing is a feature of ostertagiasis. Lymph pepsinogen levels

were elevated in agreement with previous reports on serum pepsinogen in subclinical ovine ostertagiasis.

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